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PATHOGENESIS OF RENAL LESIONS IN WEANLING AND YOUNG ADULT RATS FED CHOLINE-DEFICIENT DIETS.

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SOME years after Best and Huntsman (1932) showed that dietary choline prevented the deposition of excess fat in the liver, Griffith and Wade (1939) reported the occurrence of a fulminating, haemorrhagic renal degeneration in weanling rats fed a choline-deficient diet. The swollen, red kidneys may attain weights twice the normal. Microscopically there is a widespread, renal cortical degeneration with haemorrhage. Griffith and Wade also noted an accompanying decrease in thymus weight, increase in splenic weight, fatty livers, and in the more severely affected animals, ocular haemorrhages. The term " haemorrhagic renal syndrome " has been given to the kidney lesions.

Christensen (1940, 1941, 1942), reporting further histological studies, found the haemorrhage restricted to the renal capsule and subjacent intertubular connective tissue. The postglomerular capillaries were involved, but the glomeruli were unaffected except in particularly haemorrhagic zones. Peripheral cortical tubules were necrotic and deeper ones degenerated. Hyaline casts filled the medullary tubules and, in the most severely affected animals, the papillary ducts. In the early stages the cortical capillaries and blood vessels, including the glomerular loops, were congested. Tubular oedema with fatty degeneration was described in some instances. Stainable fat droplets in the lumina of the renal vessels were also reported by Christensen. Calcification of necrotic tubules developed in animals fed the hypolipotropic diet ten days or more. György and Goldblatt (1940) noted that the initial lesions were in the tubules. The glomeruli were affected only in the terminal stage. They also studied the kidneys of animals surviving the acute period without change of diet. Engel and Salmon (1941) confirmed the histological observations of the previous investigators. They described haemorrhagic foci in heart muscle, adrenal cortex and frequently in the lungs. Congestion of lumbar and sacral lymph nodes was seen in many instances. These investigators found the spleen sometimes swollen and bright red, sometimes shrunken and pale. The xanthydrol reaction was positive in sections of brain and kidney.

Handler (1946) observed that while few adult rats developed renal changes on choline-deficient rations, those subjected to unilateral nephrectomy uniformly

showed lesions during the period in which the remaining kidney hypertrophied. Only one of twelve unilaterally nephrectomized adult rats placed on a choline-deficient regimen two weeks after the operation developed renal damage. Gualandi, d'Erano and Campana (1947) showed by chemical estimations that the total renal lipids increased in rats on hypolipotropic diets. Histologically they observed a fatty turbid degeneration affecting some of the proximal convoluted tubules. These chemical and histological findings have been confirmed in this laboratory (Ridout, 1948, personal communication ; Hartroft and Best, 1947). Wachstein (1944, 1946) found alkaline phosphatase depleted in the damaged tubules with some increase in small cortical vessels and glomeruli. Lipase and acid phosphatase activity were also reduced in the affected tubules, the latter enzyme to only a slightly less amount than normally present.

Sobin and Landis (1947) found the blood pressures of rats unaltered during the acute and chronic stages of choline deficiency. They concluded that these renal lesions do not belong to that group which frequently produces hypertension, but instead to that which generally has no effect on blood pressure, e.g. the nephroses. Their results confirm earlier blood-pressure studies on similar animals by Chilean investigators (Honoratio and Vadillo, 1944). Referring to an unpublished paper by Sobin and Kinney, Sobin and Landis (1947) state that the cortical tubular necrosis may not be a primary effect of renal metabolic abnormality, but rather a secondary mechanical result of extensive renal vascular derangement.

Dessau and Oleson (1947) found that daily subcutaneous injections of atropine sulphate into choline-deficient weanling rats decreased the incidence of haemorrhagic kidney. They came to the same general conclusion regarding the effect of renal decapsulation. They suggested that renal vasospasm may play an important role in the tubular necrosis characterizing the condition. Baxter and van Slyke (1948) concluded that the underlying disturbance in the haemorrhagic renal syndrome was in the blood supply to the affected tubules. They did not establish its immediate cause. They found that renal decapsulation did not yield conclusive evidence of protection.

Deane and Olson (1948) reported morphological evidence of stimulation of the adrenal cortex (zona glomerulosa) in rats exhibiting the haemorrhagic renal syndrome. Adrenal cortical hypertrophy was associated with acute thymic atrophy and a progressive increase in ketosteroid secretion, both of which regressed with renal recovery. They concluded that renal dysfunction caused the adrenal cortical stimulation, and that this and the thymic involution were part of the " adaptation syndrome " (Selye, 1946).

This paper concerns the pathogenesis of the renal lesions of (*a*) weanling rats and of (*b*) young adult rats deprived of dietary choline for varying periods.

METHODS.

Weanling albino rats of the Wistar strain weighing 30 to 45 g. were fed a choline-deficient diet (Lucas, 1948, personal communication) for seven days. Groups of five were killed daily. Older animals weighing approximately 150 g. were placed on the same dietary regimen for periods up to seven months. Pairs were killed twice weekly during the first three months, and groups of five at monthly intervals thereafter.

Transversely cut, frozen sections (10μ) of formalin-fixed kidney tissue of all animals were stained with Sudan IV and haematoxylin. Paraffin sections (3μ) of additional Helly- or Boiun-fixed material from each rat were routinely stained with haematoxylin and eosin, and by Masson's trichrome method. Selected sections were specially stained to demonstrate reticulin, basement membrane, elastic tissue, mitochondria, haemosiderin or calcium. Alcohol-fixed material was stained for glycogen and by Gomori's (1939, 1945) methods for demonstration of alkaline and acid phosphatase and of lipase. Stainable lipid in frozen sections was subjected to the series of histochemical tests recommended by Lison (1936).

Renal vascular patterns were specially demonstrated in a series of choline-deficient weanling rats killed in groups of four at daily intervals for one week. The renal arterial system after perfusion with saline was injected under physiological pressure *via* the beating left ventricle with a filtered, diluted colloidal suspension of carbon (Higgins American India-ink). Alcohol-dehydrated, 300μ transverse, frozen sections of these injected kidneys after formalin-fixation were cleared in benzyl benzoate according to a method previously reported by the author (Hartroft, 1941). Frozen sections (50μ) of these injected kidneys stained with Sudan IV proved of particular value.

The day-by-day development of the haemorrhagic renal syndrome did not fall into sharply demarcated stages. For ease of description, however, the lesions of the cortical tubules are discussed under two headings—a primary period, characterized by intracellular formation of sudanophilic droplets, and a secondary terminal stage, with capillary ischaemia and tubular necrosis and haemorrhage. The pathogenesis of the renal lesions of older rats is considered under a special heading.

The Pathogenesis of Haemorrhagic Kidney.

The initial stage.—Sudanophilic droplet formation just within the peripheries of the proximal convoluted tubule cells (Fig. 5 and 6) was consistently the earliest lesion detected. Increased diameters and diminished lumina of these tubules resulted from swelling of their epithelial cells (Fig. 2). This resembled but surpassed "cloudy swelling" both in degree and extent. Only primary convoluted segments, which frequently compressed intervening secondary ones, were specifically affected (Fig. 2). This initial stage was observed from the second to fifth experimental days. Increased tubular diameters in stained sections were constantly associated with a corresponding decrease of the lumina of the intervening capillaries in cleared sections of similar kidneys injected with ink (Fig. 11 and 12).

The final stage.—Groups of necrotic tubules (Fig. 3 and 4) alternating with others containing large amounts of stainable fat (Fig. 7 and 8) were initially observed on the fifth to seventh experimental days. Antedating necrosis, small fat droplets were seen intracellularly in all the primary tubules. With onset of necrosis of some tubules, heavy deposits of fat appeared in others. Patchy alternation of dead tubules and others filled with fat is characteristic of this stage. As visualized in cleared slices of injected kidneys, failure of ink to penetrate the peripheral portions of the lobules adjacent to the interlobular vessels was the initial sign of obstruction of cortical capillaries (Fig. 13 and 14). Areas of capillary ischaemia rapidly advanced toward the centres of the lobules (Fig. 15 and 16). Stained sections show that the distribution of the patchy tubular

necrosis corresponds to the areas of capillary ischaemia (Fig. 19). In the final stage nearly the entire cortical capillary bed is obstructed (Fig. 17 and 18). Even in the last of these preparations glomerular injection is little impaired. Tubular necrosis visible in ordinary sections is now widespread (Fig. 4 and 20).

The portions of the capillary plexus remaining patent have been shown to supply those central areas of the lobules in which tubules with the largest amounts of fat were found. With the first appearance of ischaemia in the peripheral regions of the lobules, tubular sudanophilia is slight but uniform (Fig. 21). At a somewhat later stage advanced sudanophilia of centro-lobular tubules which are surrounded by injected capillaries is in contrast with necrotic tubules of the peripheral regions of lobules. These latter tubules are surrounded by obstructed capillaries. Stainable fat in areas of tubular necrosis is not greater than that seen in the previous stage (Fig. 22). Loss of nuclear staining can be demonstrated in the tubules of non-injected regions (Fig. 23 and 24). In the intervening areas fat-laden tubules showing no evidence of necrosis are surrounded with ink-filled capillaries.

Tubular casts.—These first appear early in the stage of cortical ischaemia. Tubules at the cortico-medullary junction are the first to exhibit casts (Christensen, 1942). In any sections in which casts were encountered, early cortical tubular necrosis was found ; the converse was also true.

Later in the ischaemic stage the number of casts, particularly in the medulla, greatly increased. They were homogeneous, structureless and strongly acidophilic, and exhibited polychromasia with Masson's stain, which was neither related to their age nor site of formation. Terminally, medullary tubules containing casts appear to be distended and to produce compression of intervening capillaries. Small amounts of stainable lipid in some casts suggested their origin in the cortex, where fatty changes in the tubules are common.

Histochemical tests.—Both lipase and alkaline phosphatase activity of the cells of the proximal convoluted tubules was diminished during the stage of cortical ischaemia (Wachstein, 1944, 1946). But these changes were not demonstrable during the initial stage of tubular swelling. Loss of enzymatic activity is therefore associated with necrosis. Glycogenic degeneration of the tubules was slight and variable, and is probably of little significance. Results of analysis of the lipid in the tubules by the procedures recommended by Lison (1936) suggested that the material was glyceride (saturated or non-saturated) or fatty acids.

Atropinization and renal decapsulation.

Atropinization.—Three groups, each of ten weanling rats matched for weight and sex, were placed on the same choline-deficient diet (Lucas, 1948). All members of Group A received 1 mg. of atropine sulphate in 0·10 c.c. of distilled water by subcutaneous injection once daily. Rats of Group B received no atropine and were pair-fed with those of Group A. Rats of Group C were offered the choline-deficient diet *ad lib.* After seven days all animals were sacrificed. Group averages of kidney weights were compared to estimate the incidence and severity of the renal lesions, which vary directly with kidney weight as shown by Griffith and Wade (1940). The averages of total kidney weights were 0·92, 0·90 and 0·91 g. for Groups A, B and C respectively. The figure for comparable normal rats is approximately 0·50 g.

Two additional matched groups each comprised of 20 weanling rats were placed on the choline-deficient diet until all died. Group D was injected daily with atropine in the same way as Group A above. Group E was not atropinized and was pair-fed with Group D. The extent of renal degeneration was measured by determining the average survival time recorded to the nearest quarter-day. The averages for the two groups were identical—8¼ days. In these experiments no effect of atropinization on the incidence of haemorrhagic renal degeneration could be demonstrated.

Renal decapsulation.—Bilateral renal decapsulation was performed on 10 weanling rats. In 10 others one kidney was decapsulated. Sham-operated and normal groups of comparable animals were pair-fed with the operated groups. All animals received the choline-deficient diet (Lucas, 1948, personal communication). Operations were performed the day prior to initiating the choline-deficient regimen. Group averages of survival times were compared to estimate the measure of haemorrhagic renal degeneration (Table I).

TABLE I.—*Survival Time, in Days, of Bilaterally and Unilaterally Renal Decapsulated Weanling Rats on a Choline-deficient Diet, Compared with Pair-fed, Sham-operated and Normal Controls.*

Animal number in each group.	Bilateral decapsulated.	Bilateral sham-operated.	Non-operated controls.	Unilateral decapsulated.	Unilateral sham-operated.	Non-operated controls.
1	*	8	8	7	*	7
2	9	8	9	7	7	8
3	9	7	9	10	8	10
4	9	7	9	7	7	9
5	12	*	9	8	*	9
6	*	10	†	8	7	12
7	10	*	9	10	8	9
8	9½	8	10	10	8	9
9	12	10	12	8	7	12
10	7	8	12	8	10	12
Group averages	9·6	8·3	9·7	8·3	7·8	9·7

* These animals died within 24 hours following operation and are not included in averages.

† This animal died on the 4th day of the experiment. At autopsy the kidneys were nearly normal, both grossly and microscopically. The cause of death was not determined. This animal's survival time is therefore not included in its group average.

The non-operated and pair-fed controls lived slightly longer than the operated groups. The latter in turn lived slightly longer than the sham-operated animals. These results confirm those of Baxter and van Slyke (1948), who found that renal decapsulation does not yield conclusive evidence of protection.

Additional injection experiments.—High pressures may force fluids into vessels occluded by spasm. Injections under approximately 300 mm. Hg pressure were made directly into (*a*) the renal artery (Fig. 25 and 26), (*b*) the renal vein (Fig. 27 and 28), and (*c*) artery and vein of the same kidney (Fig. 29 and 30). India-ink was used as described previously, and the kidneys were all in the late stage of cortical tubular ischaemia. In every instance all portions of the renal vascular tree were completely injected, with the exception of the post-glomerular capillary plexus in the cortex and a few necrotic glomeruli beneath the capsules.

Perfusion of kidneys in the living animal with a potent vaso-dilator may relieve vasospasm, opening the way for penetration with ink, thus modifying the previously described vascular patterns. Five animals in the terminal stages of haemorrhagic kidney were perfused *via* the left ventricle with 0·5 per cent sodium nitrite (suggested by Prof. Normand L. Hoerr, Western Reserve University, Cleveland, U.S.A.), at physiological pressure, the right renal pedicle being clamped. The nitrite solution was washed out with normal saline; the clamp on the right renal pedicle transferred to the left and saline perfusion recommenced. The left renal pedicle clamp was removed, and ink injected simultaneously into both kidneys in the usual manner. The saline-perfused right kidney served as a control for the nitrite-perfused left. Comparison of vascular patterns in cleared slices of all kidneys of animals so treated revealed little if any differences. All patterns were similar to those already illustrated in Fig. 17 and 18.

The results of these additional injection experiments suggested that renal vasospasm is not a factor in the production of haemorrhagic kidney.

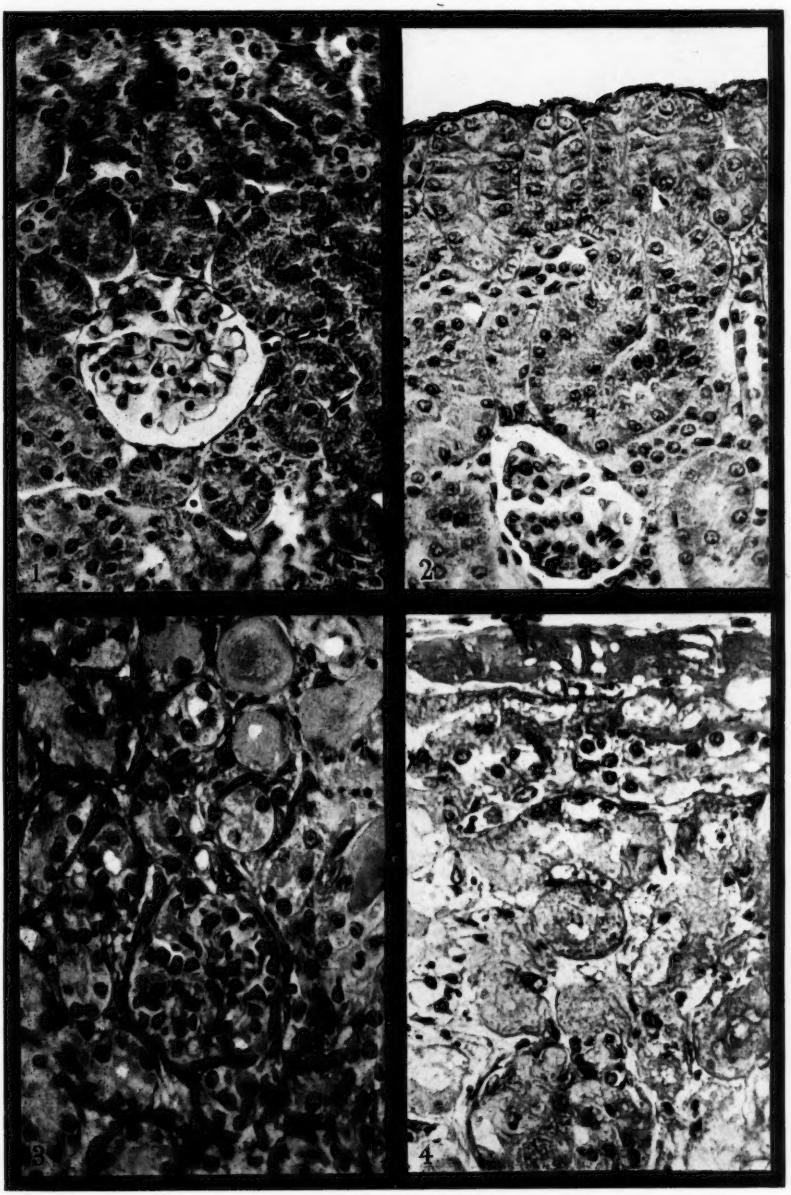
The Pathogenesis of Cortical Tubular Atrophy in Young Adult Choline-deficient Rats.

Many of the reported observations concerning the renal lesions of choline-deficient rats (Christensen, 1942; György and Goldblatt, 1940; Engel, 1942; Handler, 1946) deal only with the acute changes characteristic of haemorrhagic kidney, and the chronic or healing stages in survivors of the condition either with or without supplementary choline added to the diet. Webster (1942) reported a form of cortical atrophy occurring in rats fed low protein, high fat diets for several months. In our experiments rats weighing approximately 150 g. fed a choline-deficient diet (Lucas, 1948, personal communication) up to seven months did not develop the haemorrhagic renal syndrome. Instead, many of the cortical tubules slowly atrophied, unaccompanied by necrosis at any stage. The surfaces of such kidneys were uniformly and finely granular, resembling those described by Webster.

Histological examination of these kidneys revealed groups of relatively normal tubules interspersed with others which were atrophied or distended (Fig. 31). The outermost glomeruli were small, and occupied a position immediately beneath the capsule in many instances. This resulted from atrophy of subcapsular tubules (Fig. 32). Occasionally the glomerular and renal capsules merged (Fig. 33).

The first detectable renal lesion in these older animals, as in the weanlings, consisted of intercellular sudanophilic droplets at the peripheries of the proximal convoluted tubules (Fig. 34 and 35). But this did not appear until after 12 to 14 days of choline deprivation. In the weanlings only 2 to 5 days were required. In the older rats swelling of the tubules was absent or slight and their lumina undiminished. Nearly a month of choline-deficiency was required before stainable tubular lipid reached appreciable proportions (Fig. 36 and 37). The most severely affected tubules were neither swollen nor necrotic, but shrunken and atrophic.

Vascular patterns demonstrated in cleared slices of such kidneys injected with ink were little altered. In sections (50μ) of these stained with Sudan IV, the lobular boundaries could be identified by the prominent interlobular vessels,



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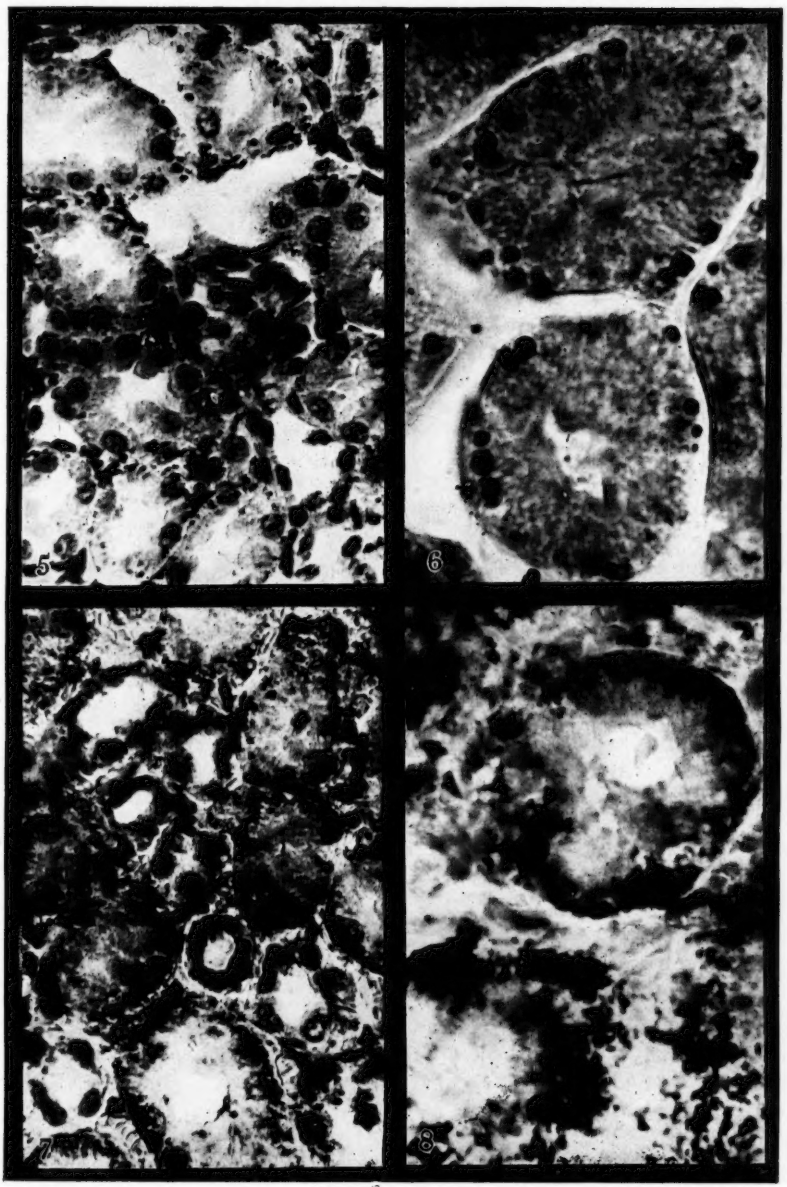

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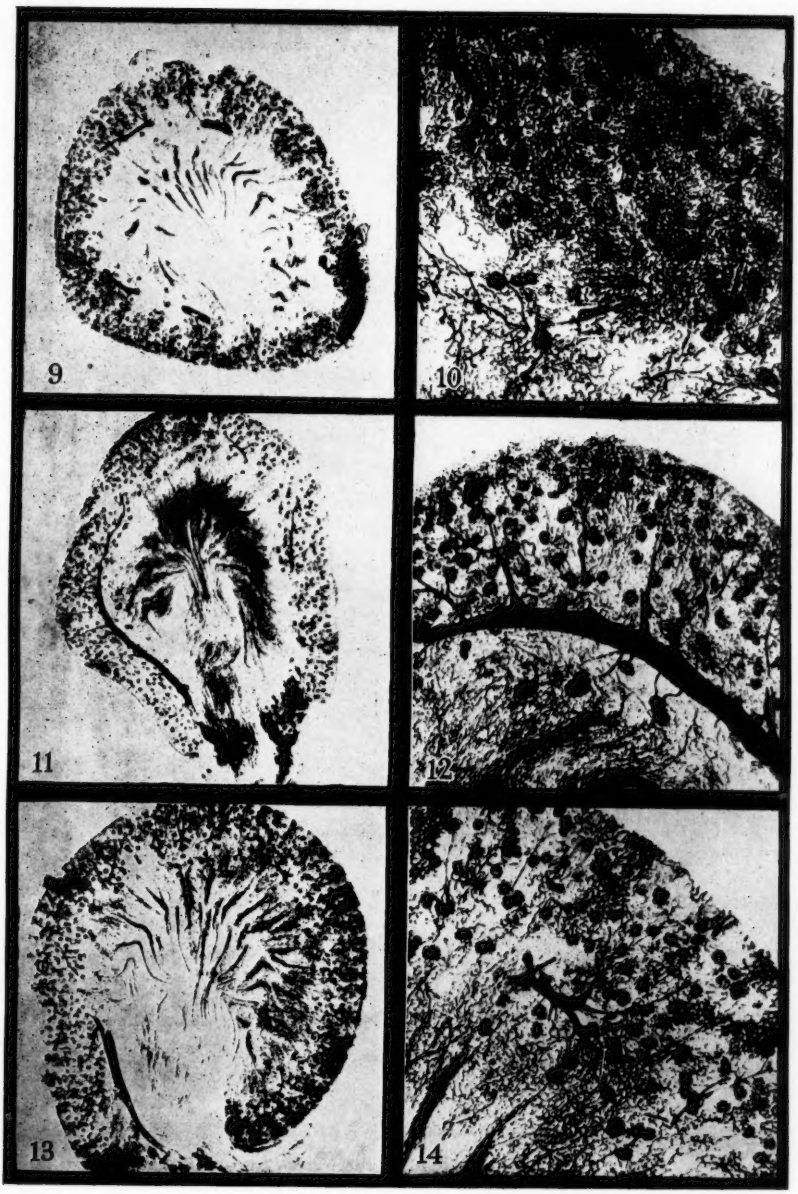

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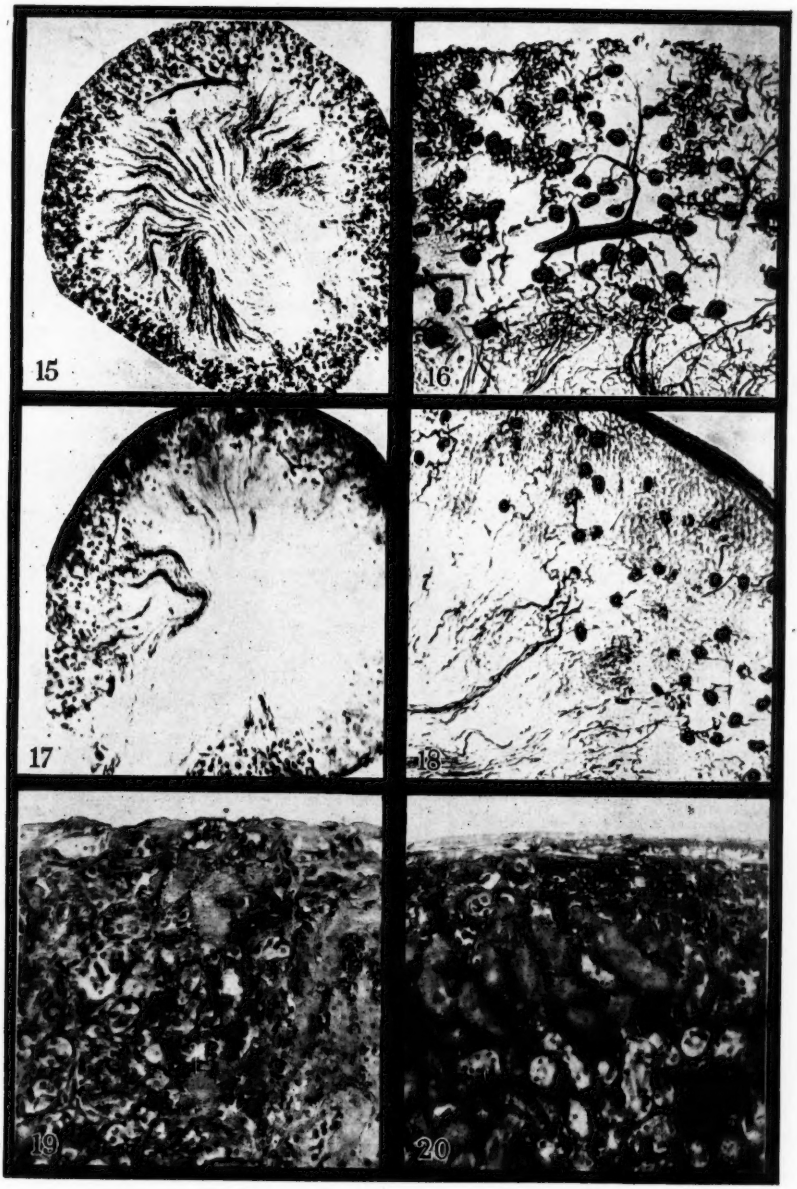

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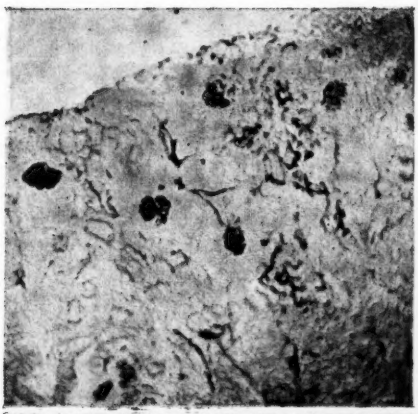

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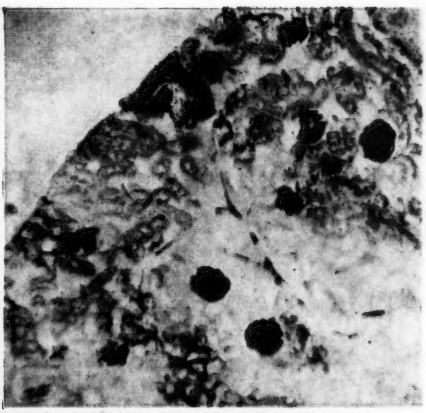

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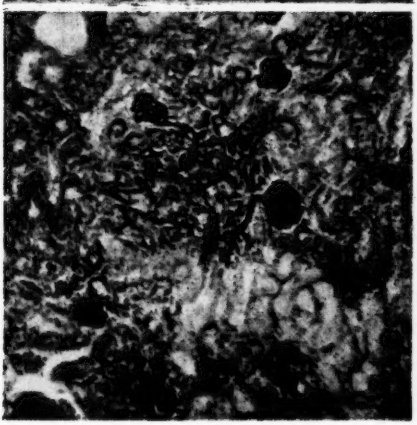

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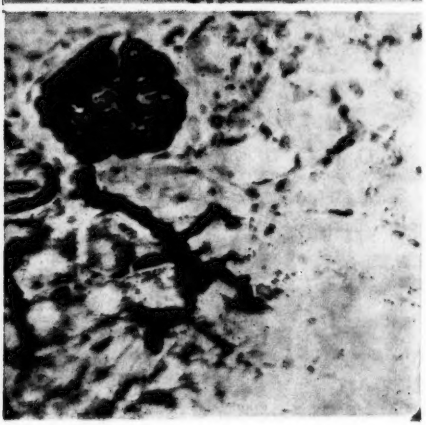

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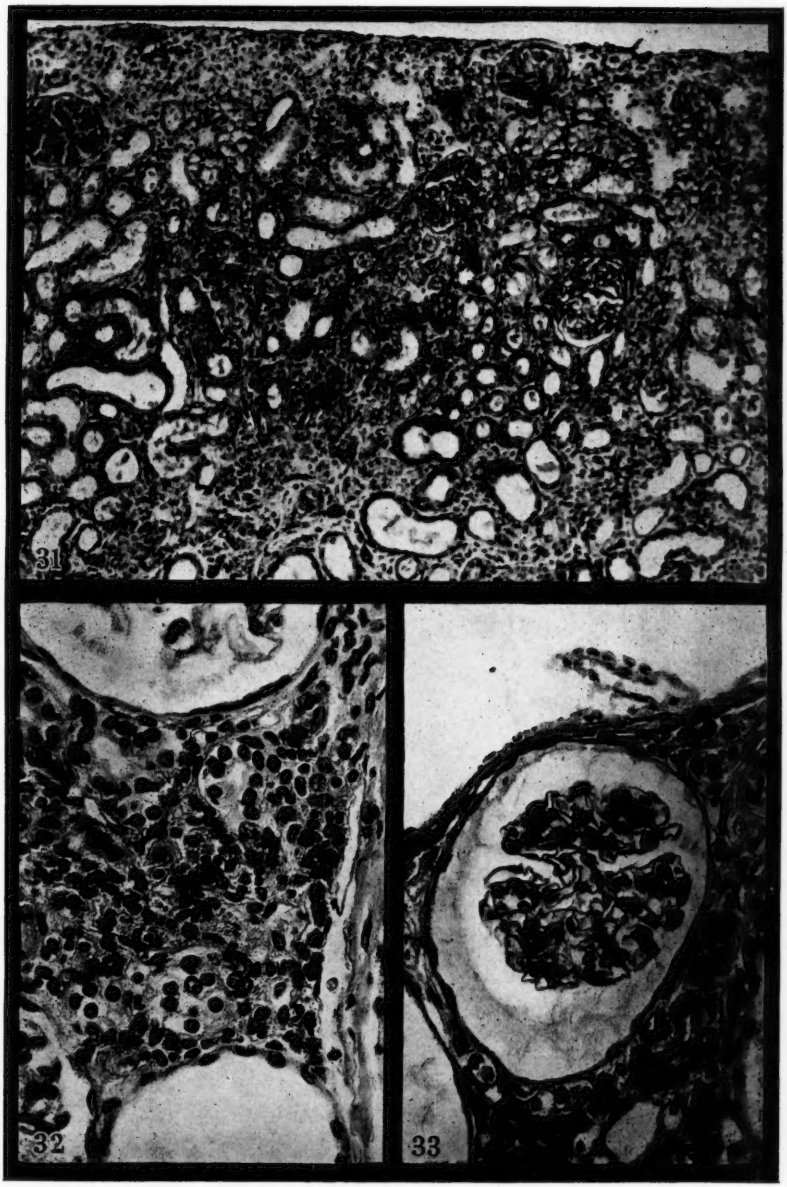

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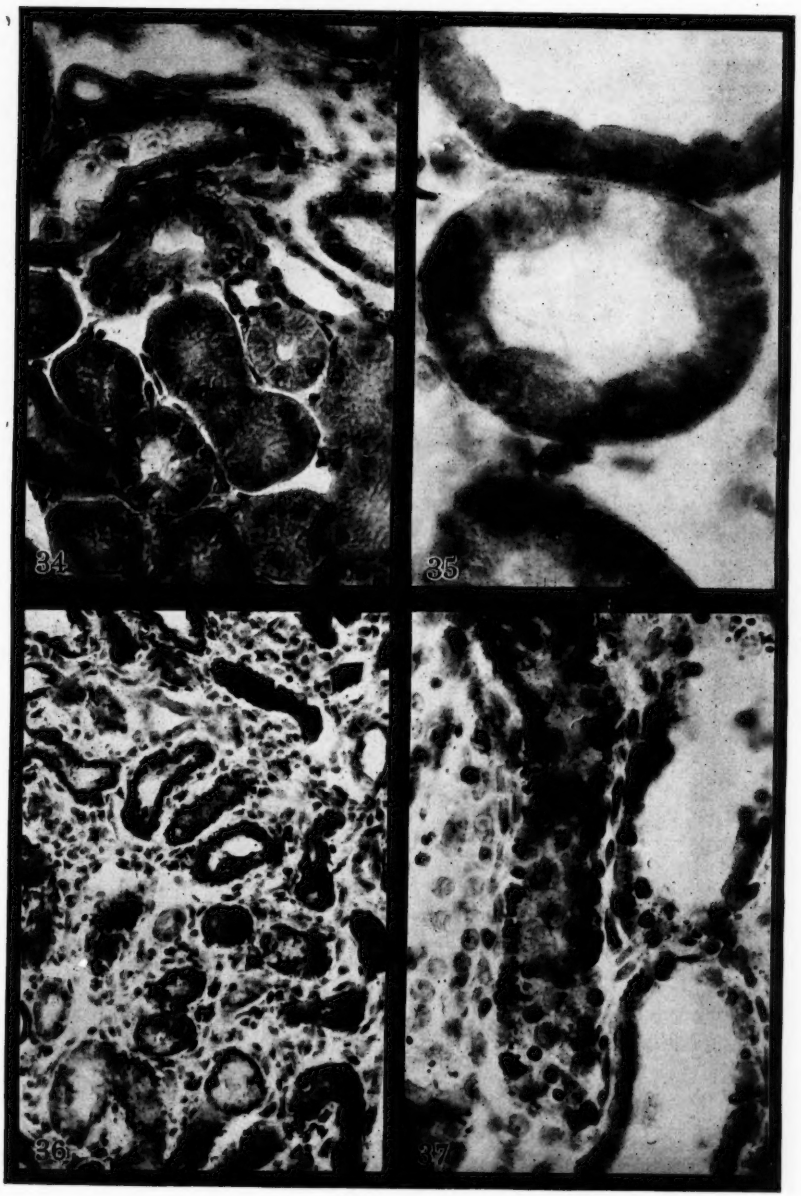

Hartroft.

EXPLANATION OF PLATES.

FIG. 1.—Cortical tubules and a glomerulus of the kidney of a normal weanling rat. Haematoxylin and eosin. × 500.

FIG. 2.—Swelling of the proximal convoluted tubules in the renal cortex of a weanling rat deprived of dietary choline for three days. The swelling involves only the proximal convoluted segments which are actually compressing two distal convoluted tubules at the top and right of the glomerulus. Haematoxylin and eosin. × 500.

FIG. 3.—Early tubular necrosis in the renal cortex of a weanling rat after five days of dietary choline deficiency. Those tubules nearest the glomerulus are not yet affected. Haematoxylin and eosin. × 500.

FIG. 4.—Generalized tubular necrosis in the renal cortex of a weanling rat in the terminal stage of haemorrhagic kidney after 7 days of dietary choline deficiency. The glomerulus at the bottom of the field is engorged and becoming necrotic. Haematoxylin and eosin. × 500.

FIG. 5.—Sundanophilic droplet formation in the cells of the proximal convoluted tubules of the renal cortex of a weanling rat on a choline-deficient diet for three days. Sudan IV and haematoxylin; frozen section. × 420.

FIG. 6.—Higher power showing the position of stainable fat droplets at the peripheries of swollen epithelial cells in the proximal convoluted tubules of a weanling rat after three days of choline deficiency. The swollen cells have encroached on the tubular lumina. Sudan IV and haematoxylin; frozen section. × 850.

FIG. 7.—Masses of tubular lipid between patches of tubular necrosis in the renal cortex of a weanling rat deprived of dietary choline for six days. Frozen section, Sudan IV and haematoxylin. × 420.

FIG. 8.—Higher power from the same kidney illustrated in Fig. 7, showing the marked increase in tubular lipid as compared to Fig. 6. Frozen section, Sudan IV and haematoxylin. × 850.

FIG. 9 to 18.—300μ cleared slices of ink injected kidneys of weanling rats.

FIG. 9.—Low-power view of the renal vascular pattern in the kidney of a normal weanling rat. × 10.

FIG. 10.—Higher power view of the renal cortex of a normal weanling rat, demonstrating the appearance of the injected post-glomerular capillary plexus. × 42.

FIG. 11.—The renal vascular pattern in a weanling rat deprived of dietary choline for three days. This is the stage of tubular sudanophilia and swelling. × 10.

FIG. 12.—Higher power detail of the cortex of kidney shown in preceding figure. Note the decreased width of the capillaries of the post-glomerular plexus. None of these are actually obstructed however. × 42.

FIG. 13.—Low-power view of kidney of weanling rat early in the stage of cortical tubular necrosis after five days of dietary choline deficiency. Note the patchy alternating pattern of the cortical capillary plexus. × 10.

FIG. 14.—Higher power of the cortex of kidney shown in Fig. 13. The capillary plexus surrounding the interlobular vessels is obstructed in each lobule, producing a peripheral lobular ischaemia. × 42.

FIG. 15.—The renal vascular pattern under low power in a weanling rat in the stage of tubular cortical necrosis and capillary ischaemia after six days of dietary choline deficiency. This represents a slightly later stage than that shown in Fig. 13 and 14. × 10.

FIG. 16.—Higher power of the cortex of the section illustrated in Fig. 15. The peripheral lobular ischaemic regions are larger than in Fig. 14. All blood leaving the glomeruli must reach the arcuate veins *via* the anastomoses between cortical and medullary plexuses, as none can reach the interlobular veins. × 42.

FIG. 17.—The renal vascular pattern under low power in a weanling rat in the terminal stage of fully developed tubular ischaemia and necrosis. Little more than the glomeruli have been injected. × 10.

FIG. 18.—Higher power of the cortex of the section shown in Fig. 17. The post-glomerular capillary plexus is now almost completely obstructed, but glomeruli are still ink-filled. × 42.

FIG. 19.—Paraffin section from the kidney illustrated in Fig. 15 and 16. The patchy distribution of the tubular necrosis corresponds to that of capillary ischaemia. Haematoxylin and eosin. × 85.

FIG. 20.—Paraffin section from the kidney shown in Fig. 17 and 18. Widespread tubular necrosis now corresponds to the widespread cortical capillary ischaemia. Haematoxylin and eosin. × 85.

FIG. 21.—50μ, Sudan IV-stained frozen section of ink injected kidney of a weanling rat early in the stage of cortical tubular ischaemia and necrosis preceding the haemorrhagic kidney of choline deficiency. Tubular lipid formation is still minimal everywhere. The peripheral regions of the lobule surrounding the interlobular artery are ischaemic. × 55.

FIG. 22.—Similar preparation as that illustrated in Fig. 21 from a weanling rat at a later stage in tubular ischaemia. The tubules in the centro-lobular regions are supplied by still-patent capillaries (ink-filled) and contain masses of sudanophilic lipid. The tubules supplied by obstructed capillaries contain no more stainable lipid than previously (Fig. 21), and are becoming necrotic. × 60.

FIG. 23.—10μ frozen section from the same kidney illustrated in Fig. 22 stained with Sudan IV and haematoxylin. Tubules surrounded by ink-filled capillaries are laden with red sudanophilic lipid. Those supplied by obstructed, non ink-containing capillaries are necrotic. × 75.

FIG. 24.—Higher power view of a region from Fig. 23 above. The origin of a portion of the capillary plexus from an efferent glomerular arteriole is shown. Tubules supplied by the first portion of the plexus originating from the arteriole are filled with lipid. This part of the plexus is patent and contains ink. Tubules supplied by more distal, obstructed portions of the plexus are necrotic. × 250.

FIG. 25 to 30.—Cleared sections of kidneys of weanling rats injected under high pressure. All rats had been deprived of dietary choline for 8 days.

FIG. 25.—Renal arterial injection. Little ink has entered the cortex. × 10.

FIG. 26.—High power of the section shown in Fig. 25. The ink has penetrated the interlobular arteries, the glomeruli and even some of the efferent glomerular arterioles, but none has entered the post-glomerular plexus. × 42.

FIG. 27.—Retrograde venous injection. There is complete injection of the medulla, but little has entered the cortex. × 10.

FIG. 28.—Higher power of cortex of section in Fig. 27. The ink has penetrated the interlobular veins and even some of the short radicles draining into them, but none has entered the obstructed capillary plexus. × 42.

FIG. 29.—Combined arterial and venous injection. Every medullary vessel is distended with ink. × 10.

FIG. 30.—Higher power of cortex of section in Fig. 29. Ink has entered both interlobular veins and arteries, but scarcely any has penetrated the cortical plexus. This demonstrates that the site of obstruction is in the plexus *per se* rather than either the arterial or venous portions of the renal vascular tree. × 42.

FIG. 31.—The microscopic appearance of the kidney of an 150 g. rat fed a choline-deficient diet for seven months. Patches of atrophied tubules are interspersed between others, which are either relatively normal or distended. Haematoxylin and eosin. × 120.

FIG. 32.—An area of advanced subscapular tubular atrophy in a similar animal. The tubules are reduced to small collections of cells still retaining, however, an acinar arrangement. Haematoxylin and eosin. × 600.

FIG. 33.—A glomerulus in the same kidney illustrated in Fig. 32 situated immediately beneath the capsule at the upper portion of the photomicrograph. The glomerular and renal capsules have fused due to disappearance of the subcapsular group of tubules. Haematoxylin and eosin. × 600.

FIG. 34.—Renal cortex two weeks after placing the 150 g. rat on a choline deficient diet. A few of the tubules contain small droplets of fat. Some show slight early distension. Frozen section stained with Sudan IV and haematoxylin. × 250.

FIG. 35.—Higher power photomicrograph of a tubule from the same section shown in Fig. 34. Small droplets of stainable fat lie at the periphery of the cells. Note that there is no cellular swelling, and that the tubular lumen is not diminished as in weanling rats (Fig. 6). × 850.

FIG. 36.—Tubular atrophy and accumulation of stainable lipid in the cortex of an 150 g. rat after 5 weeks on a choline-deficient diet. Frozen section stained with Sudan IV and haematoxylin. × 250.

FIG. 37.—Higher power photomicrograph of an atrophic lipid-laden tubule from the same section shown in Fig. 36. × 700.

and showed that the tubules containing fat were most frequently in the centre of the lobule and beneath the capsule. Some tubules were atrophied while adjacent ones dilated (Fig. 40), and these eventually achieved diameters twice that of normal or greater (Fig. 31).

Tubular atrophy occurred more rapidly than shrinkage of surrounding basement membranes, which were thrown into folds. When tubular atrophy was complete, only loops of wrinkled basement membrane remained.

In the older animals the tubular lipid initially formed was analysed according to the method of Lison (1936), with results similar to those obtained in the weanlings. Lipid in atrophied tubules at a later stage gave positive reactions for cholesterol. Alkaline phosphatase and lipase in such tubules were also reduced or absent, but not before the first appearance of stainable fat.

Ceroid (Lillie, Daft and Sebrell 1941) could be demonstrated in the cirrhotic livers of the young adult rats fed the choline-deficient diet for 7 months. Only traces could be found in their kidneys. Ceroid was almost completely absent from all organs, including the livers, of similar rats fed the same diet for 7 months if a vitamin E supplement was added as suggested by Pappenheimer and Victor (1946).

DISCUSSION.

Tubular sudanophilia.

The initial stages of the renal lesions of choline-deficiency in both weanling and 150 g. rats are identical. They consist of intracellular formation of sudanophilic droplets in the proximal convoluted tubules. The rapid appearance of the droplets in weanlings may be responsible for the associated tubular swelling, for this is absent in older rats, in which such droplets form slowly. Obstructed capillaries between the swollen tubules in weanlings result in ischaemic necrosis and haemorrhage. In the absence of tubular swelling in older rats these results are not observed. This suggests that the *rate* of formation of stainable lipid in the tubules determines the eventual form of the renal lesions. There is evidence (Perlman and Chaikoff, 1939; Boxer and Stetten, 1944) that the turnover rates of the choline-containing phospholipids of liver and kidney are related to dietary choline. The demand for choline varies directly with the growth rate. This may help to explain the rapid appearance of tubular lipid in young rats with restricted choline-intake.

Constant association of stainable fat in the renal tubules with low levels of choline in the diet of all rats studied suggests a direct relationship between the two. In weanlings neither the rate of appearance nor the extent of necrosis of the tubules bears any direct relationship to the amount of fat which appears. On the contrary, those tubules which survive the longest accumulate the most fat (Fig. 21 and 22). This suggests that the formation of the visible fat is associated with a continuing although perhaps altered metabolism of the tubule-cells which are handicapped by an insufficient supply of dietary choline.

Following obstruction of the terminal portions of the cortical capillary plexus in the weanling choline-deficient rats, all blood leaving the glomeruli must be drained *via* the remaining patent capillaries. The tubules which the latter surround are then actually hyperaemic. This may intensify the effect of the choline-deficiency in these surviving tubules.

The slower formation of sudanophilic lipid in the tubules of older rats is not

associated with tubular swelling, ischaemia and necrosis. The stainable intracellular fat appears to interfere with tubular function, for the morphological lesion which follows it is suggestive of " disuse atrophy." If such is indeed the cause of the shrinkage and eventual disappearance of the affected nephrons, it would not be expected to play a part in the pathogenesis of haemorrhagic kidney where the rapid necrosis overshadows all other changes.

Subcapsular haemorrhage.

Obstruction of the drainage of the cortical plexus into the interlobular veins results in distension of those vessels situated proximally. This is most marked beneath the capsule, where they are not limited on all sides by swollen tubules. At this potentially weak site the distended capillaries rupture with haemorrhage into and beneath the capsule. This first overlies the central portions of the renal lobules, but soon spreads in each direction and becomes continuous.

The TOC-mechanism.

It has been suggested in the foregoing that the cause of cortical ischaemia in haemorrhagic kidneys of choline-deficiency is capillary compression between swollen tubules. We have termed this mechanism *TOC*—tubular obstruction of capillaries.

Evidence for the *TOC*-mechanism resulting from the special injection studies has been presented above. The importance of this mechanism is further supported by lack of capillary ischaemia in the absence of tubular swelling in older choline-deficient animals. Other points in favour of such a concept include the distribution of the initial sites of capillary ischaemia, and the characteristics of the resultant renal vascular pattern compared to those in proven renal vasospasm. These last two points will be discussed briefly.

Gradually increasing pressure of extra-capillary origin on a plexus should first arrest the flow of blood at its terminal venous end, where intra-capillary pressure is lowest. With increase of the extra-capillary pressure obstruction of the plexus would spread toward the arterial end, where the blood is under slightly higher pressure. It has been shown (Fig. 14 and 16) that this is the sequence of events in the renal cortex of haemorrhagic kidneys. The capillary ischaemia spreads from the lobular periphery to the efferent glomerular arterioles. The swollen proximal convoluted tubules demonstrated in stained sections (Fig. 2) are therefore a suspected source of the extra-capillary pressure.

Renal vascular spasm, as shown by the Oxford investigators (Trueta, Barclay, Franklin, Daniel, and Prichard, 1947), is characterized by ischaemia of the entire outer two-thirds of the cortex affecting both glomeruli and capillaries. This is not preceded by spreading patches of ischaemia as is seen in the haemorrhagic kidney. The morphological result of prolonged vasospasm is a bilateral cortical necrosis involving not only tubules but also glomeruli. This is in sharp contrast to the renal vascular pattern in the haemorrhagic kidney, where glomerular ischaemia and necrosis are late and inconstant findings, and are overshadowed by the earlier more specific tubular lesions.

TOC-mechanism in renal conditions other than haemorrhagic kidney.

" Cloudy swelling " of renal tubules has long been recognized as a common form of degeneration. As an indication of altered cell metabolism it has been considered the product of any toxaemia resulting from infection, inorganic

poisons, or toxins of endogenous origin in such conditions as jaundice and diabetes (Boyd, 1947). That cloudy swelling may progress to actual tubular necrosis is equally widely recognized. The mechanism by which it does so, in some instances at least, may be that of *TOC*. Further investigation is indicated.

In acute renal conditions, where cortical tubular necrosis is unaccompanied by equal involvement of glomeruli, *TOC* may be the immediate cause of necrosis. Where glomeruli and tubules are equally involved, the presence of vasospasm is more strongly suggested.

In a recent review Leiter (1948) has pointed out that there is insufficient available evidence that the "tubulo-vascular syndrome" of Maegraith, Havard and Parsons (1945), characterized clinically by oliguria, anuria and uraemia, can be explained by a direct nephrotoxic action of various hypothetical agents. The mechanism here may be *TOC* following some initial disturbance of the metabolism of the tubular cells. Such would fit in well with data afforded by studies of tubular function and renal blood-flow in these cases.

A concept somewhat similar to *TOC* has recently been propounded by Glynn and Himsworth (1948) for the liver. They have shown that the sinusoids of the terminal portion of the hepatic intralobular circulation around the central vein may be obstructed by swollen liver cells in carbon tetrachloride poisoning. The resulting ischaemia, rather than a direct toxic action of the chemical on the liver cells, is considered responsible for the necrosis.

The two types of renal tubular lesions, necrotic in weanlings and atrophic in young adult rats, have been presented as separate, distinct entities. This was the case in the animals studied. But in rats intermediate in size between these two groups, dietary choline deficiency may produce both types of lesions. In such kidneys necrosed tubules at the peripheries of the lobules lie adjacent to central atrophic tubules.

If the animals survive sufficiently long the end-result of either patchy tubular necrosis or atrophy is similar. The tubules disappear and are replaced by scarred, contracted areas. Differentiation of the lesions may be sometimes aided by suitable histochemical tests. Detection of haemosiderin, indicating previous haemorrhage, strongly suggests tubular necrosis rather than atrophy. Deposition of calcium salts is frequently observed at the sites of tubular necrosis, but has not been demonstrated following atrophy.

SUMMARY.

1. The first lesion in both the acute haemorrhagic kidney of weanling rats and the atrophic tubular degeneration in young adult animals resulting from diets low in choline consists of the intracellular formation of small droplets of fat in the proximal convoluted tubules of the renal cortex.

2. The fat droplets appear after two to five days of dietary choline-deficiency in weanling rats. The appearance of the fat is associated with generalized tubular swelling. Evidence has been presented that this swelling compresses the cortical capillary plexus, producing tubular ischaemia and necrosis. This mechanism has been referred to as tubular obstruction of capillaries—*TOC*.

3. Young adult rats receiving diets low in choline for periods as long as 7 months also develop cortical tubular lesions in the kidneys. But these are atrophic rather than necrotic in nature. These lesions are also preceded by

the formation of intracellular lipid, but in the older animals it requires from 12 to 14 days for the lipid to appear. There is no evidence of tubular swelling, or any alteration in the renal vascular pattern.

4. The atrophic tubular lesion in 150 g. rats appears to be a " disuse " type. It is suggested that this is the result of interference with function produced by the accumulation of stainable fat in the cells of the affected tubules.

The author is greatly indebted to Prof. C. H. Best, Banting and Best Department of Medical Research, University of Toronto, and to Prof. William Boyd, Department of Pathology and Bacteriology, University of Toronto, for their many stimulating suggestions and kindly criticisms throughout the course of this work.

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THE STAINING OF THE DUODENAL MUCOSA OF RATS FOLLOWING THE INJECTION OF SOLUTIONS OF TANNIC ACID.

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In the course of experimental investigations of the toxicity of tannic acid a peculiar staining of the duodenum was noted in rats killed after the subcutaneous injections of solutions of tannic acid. These observations were not recorded in the original paper (Barnes and Rossiter, 1943), but have been followed up in recent experiments which are described below.

Briefly it can be stated that within 7 days of a single subcutaneous injection of 1 ml. of 20 per cent tannic acid, the duodenum of the white rat shows a very striking and characteristic black staining of the mucosa. No evidence has been found of any damage to the duodenum, but the fact that the staining is found only in this locality suggests that its development may in some way be connected with a special function of this part of the intestinal tract.

An account of the experimental investigations is given, and followed by a discussion of the possible explanation of the phenomenon.

EXPERIMENTAL METHODS.

Animals.—Albino rats—male and female—have been used. Adults weighing 150–200 g. were used for the majority of the experiments. Young rats just weaned (50–60 g.) were used in some of the feeding experiments.

White mice and cross-bred guinea-pigs and rabbits were used in a few experiments.

Special feeding, etc.—Animals maintained on an " iron free " diet were housed in glass cages with perforated zinc tops coated with " bakelite " paint. The diet consisted solely of bread and milk or dried milk paste.

Animals given extra iron to drink were given the appropriate solutions containing 1 mg. Fe/ml. as the sole source of drinking fluid. The solutions of iron salts were consumed readily by the rats.

Operative procedures.—These were carried out under nembutal and ether without aseptic precautions. The gastro-enterostomy operations were the simple anterior type of anastomosis with double suture lines made with fine catgut (eye sutures) dressed to the needle.

Histological examination.—The animals were killed with chloroform and the tissues fixed in formol alcohol and embedded in the usual way.

Routine staining was with haematoxylin and eosin, and the iron stain used was 4 per cent potassium ferrocyanide with hydrochloric acid—the Prussian blue reaction. Sections were also examined unstained, and a few after staining with Heidenhain's iron haematoxylin and carmine.

Materials.—Tannic acid (Allen & Hanbury) solutions in water were made up for each experiment. *Sodium tannate* (BDH) and gallic acid (BDH) were

also used, the latter as a saturated solution. Iron salts were of the ordinary commercial grade freshly made up as solutions for drinking.

EXPERIMENTAL RESULTS.

1. *Effect of a single subcutaneous injection of* 20 *per cent tannic acid.*

Adult white rats on a stock diet were given a subcutaneous injection over the back of 1 ml. of 20 per cent tannic acid. Animals killed from the 4th day after the injection show a distinct black staining of the duodenal mucosa. The intensity of the staining increases for periods of 10–20 days after the injection remains stationary and then gradually fades. It is still visible 50 days after the injection. One animal killed 70 days after the injection showed some staining. The staining begins abruptly on the duodenal side of the pylorus, and here is at its maximum intensity. It gradually fades off into the ordinary pink mucosa of the jejunum and extends for 8–12 cm. from the pylorus.

This, briefly, is the fundamental observation, and the rest of the paper describes the characteristics of this staining and experiments carried out in an effort to elucidate the factors governing its production. The staining is seen nowhere else in the intestinal tract nor in any other organ except, in certain cases, the mesenteric lymph nodes. The pigment in these glands has obviously been carried there from the duodenum. A dense eschar develops in the skin over the site of injection.

Fig. 1 shows the duodenum of a rat killed 10 days after the injection of 1 ml. 20 per cent tannic acid subcutaneously lying alongside the duodenum from a normal rat. Fig. 2 shows the nature of the pigmentation in greater detail.

2. *Amount of tannic acid, route of administration and time taken for the stain to develop.*

Subcutaneous injection.—A single dose of 1 ml. of 10 per cent tannic acid will produce well-marked staining in an animal which is killed 15 days later. 1 ml. of 5 per cent tannic acid produced very faint staining, and 1 ml. of 2·5 per cent tannic no visible staining after the same period. 0·1 ml. of 20 per cent tannic acid produced only a faint staining.

A few animals were given 5 injections of 1 ml. of 20 per cent. tannic acid at weekly intervals. Further injections could not be given, and the animals were killed because of the extent of the scarring over the back. These animals showed staining of the duodenum which was much more intense but no more extensive than after a single injection. They also showed staining of the mesenteric lymph glands with characteristic black pigment deposits in the periphery of the glands. One animal had a well-marked early cirrhosis of the liver.

EXPLANATION OF PLATES.

FIG. 1.—Duodenum of a rat killed 10 days after the subcutaneous injection of 1 ml. 20 per cent tannic acid compared to that from a normal rat. × ⅔.

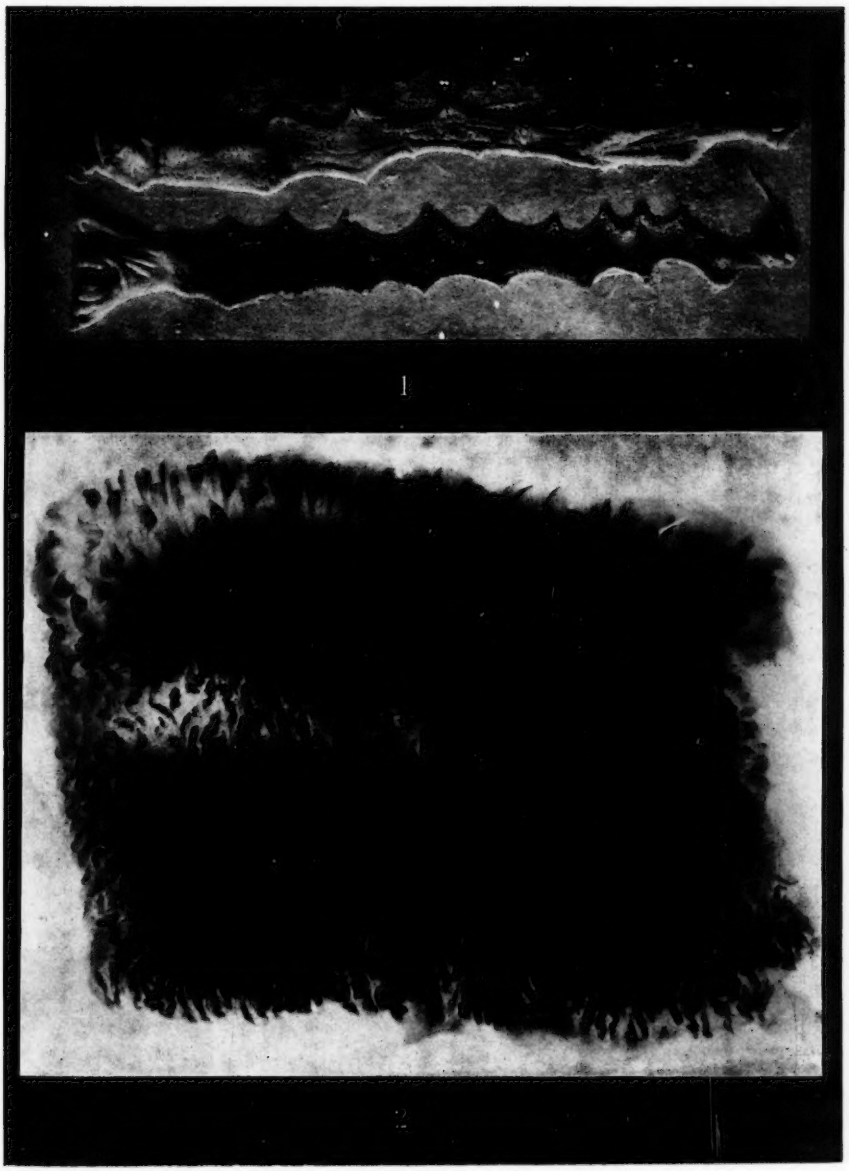
FIG. 2.—" Close-up " of a section of the duodenum shown in Fig. 1.

FIG. 3.—Distribution of pigment in villi of rat's duodenum (unstained section).

FIG. 4.—High power of section in Fig. 3 showing relation of pigment to wall of blood vessels.

FIG. 5.—High-power view of duodenum villus stained for iron. The dark stain is bright blue in the sections.

FIG. 6 and 7.—Unstained section through a mesenteric lymph gland showing the macrophages filled with pigment in the sinuses of the gland.



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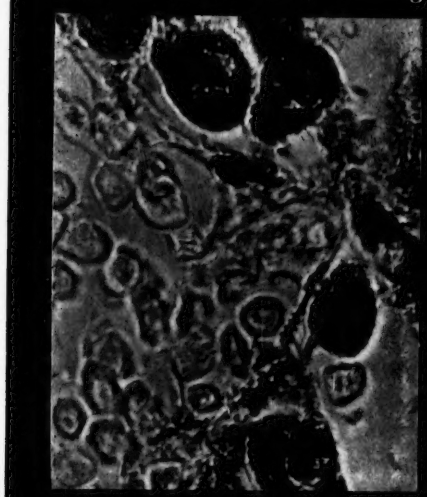

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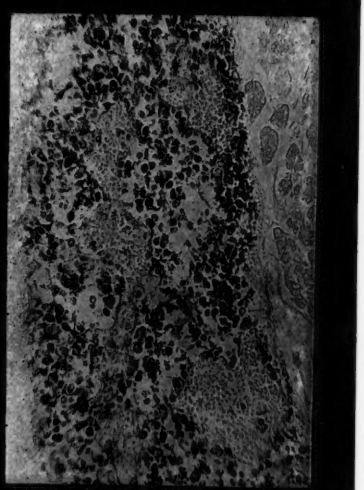

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Intravenous injection.—Only a few recent experiments have been done using this route. A single injection of 10 mg. produced no staining in 5 days. A second injection of 10 mg. produced a little in a further 5 days. Daily injection of 2·5 mg. for 10 days produced a very well-marked staining at the end of that period.

Development of the duodenal staining.—After a single subcutaneous injection of 1 ml. of 20 per cent tannic acid the staining does not become visible to the naked eye for at least 4 days, and it is then just visible on inspection with a hand-lens of the freshly opened duodenum. By 10 days the staining is clearly visible in the unopened duodenum. Fading takes place gradually over a period of several weeks.

3. *Attempts to produce staining with other agents and in other species.*

1–2 ml. of 20 per cent sodium tannate and 1–3 ml. saturated gallic acid solution injected subcutaneously in the white rat produce no staining of the duodenum.

A single injection of 2 ml. of 20 per cent tannic acid subcutaneously produced no staining in the guinea-pig. Three injections of 1 ml. of 20 per cent. tannic acid given at weekly intervals produced a faint but characteristic staining in the duodenum.

A single rabbit given a corresponding amount of tannic acid showed no staining. Groups of mice injected with 0·1 ml. of 20 per cent tannic acid showed staining in some cases, none in others, and about one-third of the animals died within 48 hours of the injection, presumably from the toxic effects of the tannic acid.

Cats and dogs have not been investigated.

4. *Histological characteristics of the duodenal staining.*

The staining is due to a brown pigment found in the tips of the villi of the duodenum. Staining begins abruptly on the distal side of the pylorus. The pigment lies beneath the epithelial layer, and in its earliest stages affects the very tip of the core of the villus. As the pigmentation develops it spreads down the villus, and after repeated injections may affect its whole length. It does not involve Brunner's glands or the submucosa. In the rat the pigment is deposited mainly on the adventitial membranes of the blood vessels as they twist up the sides of the core of the villus (Fig. 3 and 4).

The staining appears to be more intense on the outer wall of the capillary. In unstained sections the pigment appears dark brown or black, and granules can also be seen within macrophages lying in the connective tissue.

In the guinea-pig there is very faint staining of the capillary walls, but much more pigment collected in the macrophages.

The pigment appears brown in sections stained with haematoxylin and eosin. In sections stained for iron with acid and ferrocyanide the pigment now appears bright blue (Fig. 5). In other words the black pigment contains iron and is almost certainly iron-tannate.

Originally every villus is affected (Fig. 3), but as the staining fades occasional villi only remain stained, and some of the pigment appears to be carried away by macrophages. This carriage by macrophages certainly takes place after excessive pigment production following repeated injections of tannic acid. Loaded macrophages can be seen crowding the sinuses of the mesenteric lymph glands (Fig. 6 and 7).

No pigmentation has been seen in the liver and spleen or in the few kidneys that have been examined. Black material can be seen in sections taken through the site of the injection, but this does not stain for iron and is almost certainly simple " tanned " protein.

The duodenum of the normal rat stained for iron by the same method shows only an occasional blue granule in a macrophage. There is no iron staining of the vessel walls.

There is no histological evidence of damage to any other cells of the duodenum, and no evidence that the changes in the capillary walls have affected the epithelial cells of the villi. Rats kept alive for up to 70 days after the injection of tannic acid have shown no evidence of functional derangement of the duodenum. Repeated doses of tannic acid do produce more intense staining in the gut, but they also result in severe liver damage, and so the general condition of the animals is disturbed for that reason, if for no other.

5. *Effect of varying the iron-content of the diet.*

The presence of the dark iron-containing pigment in the villi of the duodenum naturally suggested that its development here, and nowhere else in the intestinal tract, resulted from the fact that iron was absorbed from the food at this point. Although this was the old belief, recent work with radio-active iron has shown that absorption takes place along the whole length of the small intestine (Copp and Greenberg, 1946).

Rats were given solutions of ferrous ammonium sulphate, ferric ammonium sulphate and colloidal iron each at a concentration of 1 ml. Fe/ml. as their sole source of drinking fluid. Such animals when injected with 1 ml. of 20 per cent tannic acid and killed at intervals showed no increase in the degree of duodenal staining nor any increase in its speed of appearance as compared to control animals on the same diet without additional iron. Young rats just weaned were fed on an iron-free diet for 10 days, and then given an injection of tannic acid and maintained on the same diet. They developed the duodenal staining just as the control animals on a normal diet. The process of absorption of iron from the diet does not therefore appear to play any part in the deposition of this iron-containing pigment in the duodenum.

6. *Effect of modifying the function of the duodenum.*

In a series of adult rats a simple gastro-enterostomy was performed by anastomosing the first part of the jejunum to the anterior wall of the stomach.

In the first group of rats the pylorus was occluded with a stout cotton suture only. Tannic acid was injected 10 days after the operation and the animals killed 14 days after the injection. In all the animals but one the cotton ligature had disappeared and both the pylorus and the enterostomy stoma were patent so that a functioning duodenal loop was formed. In these latter animals the duodenal loop was well stained (Fig. 8), while in the animal in which the pylorus was still occluded there was no staining of the duodenal loop (Fig. 9) and only some faint staining round the stoma.

A second group of animals then had a gastroenterostomy performed after division of the pylorus between ligatures.

In these animals there was no staining except the little that occurred round the mouth of the stoma. In one animal the division had been made beyond the

pylorus so that a small stump of the duodenum remained on the end of the stomach. This was intensely stained, while the blind end of the loop just beyond showed no staining (Fig. 10).

These observations suggest that the development of this pigment is in some way connected with the normal functioning of the duodenum.

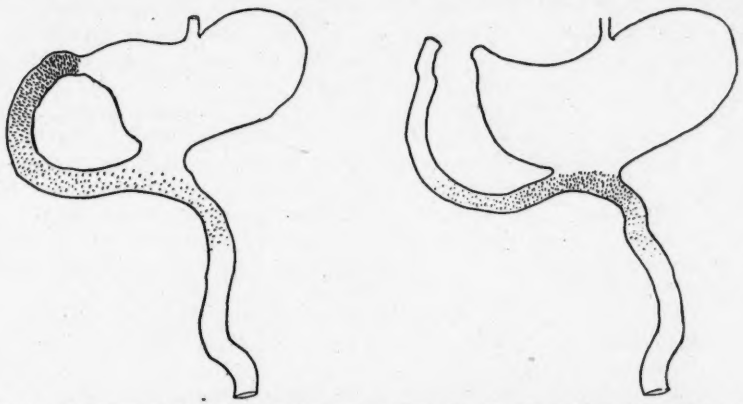

FIG. 8.—Distribution of pigment in duodenum after the reopening of the pylorus following a gastro-enterostomy.
FIG. 9.—Distribution of pigment after division of the duodenum and establishment of a gastro-enterostomy.

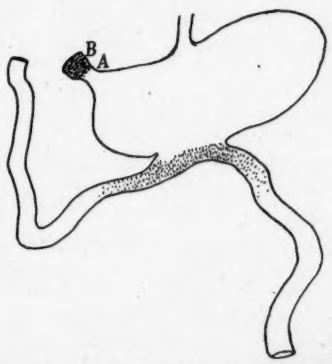

FIG. 10.—Staining in the duodenal stump left attached to the stomach after gastro-enterostomy.
A = Pylorus. B = Duodenal stump.

The development of the duodenal staining was also observed in rats that had the common bile duct tied immediately before injection of the tannic acid. Many of these animals died within the first 7 days. Of the 12 animals observed it can be said that staining does develop after ligation of the bile duct, but it takes a little longer to do so, and is not so intense as in control animals that had the abdomen opened and the gut handled but no ligature placed round the bile duct.

DISCUSSION.

No immediate explanation of the selective staining of the duodenum after the subcutaneous injection of tannic acid seems to offer itself.

Tannic acid injected under the skin will immediately combine with the tissue proteins to form tannic acid protein complexes. These are stable in acid and neutral media but dissociate in the presence of alkali. Such complexes would almost certainly be absorbed slowly, and in this connection it should be noted that if the site of the injection be excised within 48 hours no staining will develop subsequently in the duodenum. It is possible that the tannic acid is liberated from this combination with protein only in the villi of the functioning duodenum.

The fact that the pigment contains iron suggests very strongly that its deposition in this part of the intestinal tract must be related to the absorption of iron from the food. There is no evidence to support this belief. The amount of pigment deposited is not affected by increasing or decreasing the amount of iron in the diet, even if associated with repeated bleeding from the tail vein. Sodium tannate and gallic acid both form black pigments with iron salts, but neither give rise to this pigment when injected subcutaneously.

If the tannic acid-protein complexes alone could react with iron, then iron-containing pigments should be found round the site of injection in the skin. No such pigments have been found.

The only conclusion than can safely be drawn is that round the walls of the capillaries in the villi of the duodenum the optimal conditions exist for the combination of iron and tannic acid both of which are probably present in very high dilutions.

SUMMARY.

1. A striking black pigmentation of the duodenal mucosa develops within 7–10 days after a single subcutaneous injection of 1 ml. 20 per cent tannic acid into the white rat.

2. The staining is due to a deposit of some iron-containing pigment—almost certainly iron tannate—on the adventitial membrane of the capillaries in the duodenal villi.

3. The staining develops within 2–3 weeks to a maximum and then gradually fades over the next 6–10 weeks. Repeated injections of tannic acid intensify the deposition of the pigment.

4. The feeding of iron in excess affects neither the rate of development nor the intensity of the staining. It will also develop in young rats on an "iron-free" diet.

5. By-passing the duodenum by a gastro-enterostomy will prevent its becoming stained.

6. The phenomenon is discussed briefly, but no satisfactory explanation can be offered.

I wish to thank Mr. E. G. Cuff, head of the Photographic Section, C.D.E.E., Porton, for the photographs and photomicrographs.

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AN INVESTIGATION OF IMMUNOLOGICAL RELATIONSHIPS BETWEEN THE VIRUSES OF VARIOLA, VACCINIA, COWPOX AND ECTROMELIA BY NEUTRALIZATION TESTS ON THE CHORIO-ALLANTOIS OF CHICK EMBRYOS.

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A GREAT deal of experimental work in animals and observations on humans have served to show the close immunological relationship which exists between strains of vaccinia virus propagated in the laboratory and the virus of variola. It has frequently been shown that variola virus on adaptation to the rabbit eventually assumes the characters of laboratory strains of vaccinia; certain current laboratory strains of vaccinia virus have been derived in this way (Horgan, 1938; Horgan and Haseeb, 1945). Precipitation and complement fixation tests, using as antigens variola extracts from cases of smallpox or vaccinia extracts from rabbits and rabbit antivaccinial serum or smallpox convalescent sera have also served to confirm the close relationship between these viruses.

Cross immunity experiments in animals have the disadvantage that variola virus, when adapted to the common laboratory animals, may lose immunological specificity. Cross immunity experiments in monkeys in which variola virus seems to produce an infection similar to that in man, are perhaps less open to this objection; such experiments show almost complete cross immunity between the two viruses (Horgan and Haseeb, 1939).

Strains of variola virus derived directly from human material readily infect the chorio-allantois of chick embryos and after repeated passage in this tissue continue to produce characteristic lesions which differ from those of vaccinia (Nelson, 1943; Downie and Dumbell, 1947). As we had available many strains of variola virus recently isolated by chorio-allantoic inoculation, it seemed that serological investigation of these strains, using the neutralization test on the chorio-allantois, might be instructive. Accordingly immune sera were prepared by inoculating fowls with several variola strains. Fowls were chosen in order to avoid the formation of antibody to chick protein which would have resulted from the immunization of other animals with virus prepared from infected chorio-allantois. Immune sera were similarly prepared against vaccinia virus, against a strain of cowpox virus derived from an infected cow and against a strain of ectromelia virus. This latter was included as the work of Burnet and his colleagues has shown that it belongs to the pox group (Burnet and Stone, 1946; Burnet and Boake, 1946; Fenner, 1948). Certain neutralization tests were also made with rabbit antisera which had been prepared against vaccinia and cowpox strains which had been propagated in rabbits. Human sera from small-

pox convalescents and from recently vaccinated individuals were also examined for comparative purposes. The results of these observations are recorded in this paper.

MATERIAL AND METHODS.

Strains of variola used.

Variola strains.—Of the strains available the four selected had been isolated by chorio-allantoic inoculation from unrelated cases of variola major which had occurred in this country from 1944 to 1947. The originally infected egg membranes or the first passage material had been dried from the frozen state and kept in sealed ampoules in the refrigerator since isolation.

Strain H was isolated in 1944 from one of the cases in the Middlesex outbreak reported by Bradley, Davies and Durante (1946). Infection in this outbreak had possibly originated in Gibraltar.

Strain G was isolated in 1945 from a patient infected in North Africa (Public Health Note, 1945).

Strain B was isolated from a haemorrhagic case in the Merseyside outbreaks in 1946 (Pierce, 1947). The patient had been infected on board a ship coming from India.

Strain L was from a case in the Staffordshire outbreak in 1947 (Smith, 1948). The first case in this outbreak was believed to have been infected in India.

Alastrim.—A strain of virus, strain J, was isolated in 1947 from crusts kindly sent us by Dr. Usher Somers from the Sudan.

Vaccinia.—The strain used to prepare antiserum in the fowl was isolated in 1946 by chorio-allantoic inoculation of vesicle fluid from a young adult who had been vaccinated with commercial lymph. The immune rabbit serum which was used in some experiments had been prepared by immunization with an old laboratory strain of vaccinia which had been passed by one of us over a period of years in rabbits only. A suspension of this virus prepared from infected rabbit skin was used in certain experiments recorded below.

Cowpox.—The strain used for immunization of fowls had been isolated from material derived from an infected cow in 1947. This strain was passed exclusively on the chorio-allantois. The rabbit antiserum had been prepared in 1938 against a strain of cowpox virus isolated from a lesion on the hand of a cowman and passed repeatedly in rabbits. A suspension of this strain prepared from infected rabbit skin was also used in some neutralization tests.

Ectromelia.—This virus was the Hampstead strain and was received from Dr. Andrewes in 1946.

Preparation of immune sera.

Living virus was used for the inoculation of fowls, the birds receiving variola suspensions being kept in strict isolation. In all cases the viruses had been passed not more than four times on the chorio-allantois since isolation from the original host with the exception of the ectromelia virus which was prepared from the fifth passage of the original material. From eight to ten membranes from 10–12-day chick embryos showing confluent takes were used to prepare virus suspensions in each case. After draining off excess fluid the membranes were shaken up in screw-capped bottles containing sterile glass beads and 10·0 ml. of M/250 citric

acid-phosphate buffer of pH 7·2. The supernatant fluid from low speed centrifugation was spun at high speed in an angle centrifuge to deposit the virus. The deposit was resuspended and again sedimented from fresh buffer in a high speed angle centrifuge. This deposit was resuspended in 10 ml. of buffer and used for injection into fowls. The virus content of each suspension was determined by inoculation of tenfold dilutions on the chorio-allantois. The titre of the suspensions varied between 10^7 and 10^9 infective doses per ml. Samples of blood were removed from each fowl before inoculation of virus. Injections of virus were given to all fowls as follows: 1·0 ml. subcutaneously, 16 days later 3·0 ml. intraperitoneally, 7 days later 2·0 ml. intramuscularly and after a further interval of 9 days 1·0 ml. intravenously. Samples of blood were removed from the wing veins for test immediately before the second and third injections and the birds were bled and killed 6 days after the last injection. The antibody content of the sera was low after the first and second injections and the results recorded below were obtained with the final samples of serum. None of the birds showed ill effects from the injections of living virus. No lesions other than single small nodules at the sites of injection were observed. Small samples of blood were taken from several of the birds at intervals from 15 minutes to 9 days after the first injection for examination for the presence of living virus. In no instance did infection of the chorio-allantois of chick embryos develop from inoculation with these samples. Faeces from certain of the birds were examined daily for virus by inoculating suspensions of faeces with penicillin and streptomycin on the chorio-allantois; these also failed to demonstrate living virus.

A control serum was prepared against an extract of normal chorio-allantoic membranes. This extract was prepared in the same way as the virus suspensions and the fowl received the same number of injections by the same routes on the same days.

The rabbits used for the preparation of vaccinia and cowpox antisera had been injected intradermally with virus propagated by dermal infection in these animals; after subsidence of the resulting skin lesions they had received repeated intravenous injections of living elementary body suspensions.

Neutralization Tests.

All sera were inactivated before use. When egg-grown virus was used the virus suspensions were prepared as for the immunization of fowls. In the tests with vaccinia and cowpox viruses propagated in rabbits relatively pure elementary body suspensions were used. Dilutions of virus suspensions were made in saline to which broth had been added to a concentration of 10 per cent. Sera were mixed with an equal volume of virus suspension and the mixtures allowed to stand 1 to 2 hours at room temperature before inoculation of 0·1 ml. volumes on the chorio-allantois of 10–12-day chick embryos. Dilutions of virus for admixture with serum were chosen on the results of preliminary titration with the object of obtaining a countable number of discrete lesions in the membranes inoculated with control or normal serum-virus mixtures. In preliminary tests three eggs were inoculated with each serum-virus mixture; in later tests four to six eggs were used. The eggs were opened again 2 or 3 days after inoculation; the membranes were cut out and placed in formol saline to facilitate counting of lesions.

TABLE I.—*Neutralization Tests with Immune Sera Prepared in the Fowl Against Viruses Propagated in the Chorio-allantois.*

Fowl sera	Number of lesions developing on membranes inoculated with serum virus mixtures. Virus suspensions.				
	Cowpox.	Vaccinia.	Ectromelia.	Variola H.	Alastrim J.
Cowpox	8, 9, 9	8, 10, 22, 36	2, 5, 5, 11, D	4, 23, 25, 44, D	5, 6, 7, 22
Vaccinia	4, 9, 15	19, 20, D, D	3, 8, 13, 25, 29	65, 66, 76, 76, 184	0, 2, 3, 56
Ectromelia	1, 10, 15	21, 33, 49, 54	2, 11, 11, 15, D	46, 48, 59, 59, 95	0, 28, 47, 64
Variola H	1, D, D	10, 21, 41, 42	3, 5, 13, 18, 55	12, 49, 73, 78, 124	7, 9, 20, 39
,, G	6, 11	—	2, 3, 34, 36, 47	—	—
,, B	3, 4	10, 16, 17, 23	3, 5, 13, 18, 20	14, 16, 17, 37, 46	1, 4, 9, 17
,, L	2, 7	—	2, 7, 12, 22, 29	—	—
Alastrim J	12, 25	14, 19, 48, 52	7, 9, 20, 59, D	3, 5, 11, 18, 21	0, 2, 5, 7
Normal and control	48, 85, 116, 123, 181	75, 81, 104, 112 170, 214, 220, 280	45, 56, 86, 95, 145, 163, 180	325, 485, 710, 725, 735, 750, 995	111, 125, 134, 184, 295, 370, 470, 640

D = Embryo died and detectable lesions not present on membrane.

RESULTS.

Neutralization of viruses grown in the chorio-allantois by immune sera prepared in fowls.

The results of five representative neutralization experiments using various egg-grown virus suspensions and undiluted immune sera from fowls are shown in Table I. In each experiment various sera were tested against one virus so that each column represents a separate experiment. Tests were made using these fowl sera against the other three variola strains; the results were substantially the same as those shown for strain variola H and have not been included in the table. The figures in this and subsequent tables, with the exception of Table II, represent the number of lesions present on individual membranes. The results with mixtures containing control or normal fowl serum have been pooled as they were substantially the same. In a few experiments in which a broth-virus mixture was included the number of the resulting lesions on membranes did not differ essentially from that produced by virus-normal fowl serum mixture. Semi-confluent takes with vaccinia and cowpox viruses were recorded as ">200" and with other viruses as ">500." The results in Table II have been expressed to the nearest whole number as percentages of pock counts produced by the immune sera as compared with normal fowl and control fowl serum; this has not been done with results recorded in Tables III to VI, as the control membranes showed semiconfluent takes and accurate enumeration of lesions was impossible. Variation in the number of lesions on membranes receiving the same inoculum as seen in Table I are well recognized by workers using this technique (Keogh, 1936; Beveridge and Burnet, 1946.)

TABLE II.—*Average Figures of Pock Counts in Table* I *Expressed as Percentages of Controls.*

Fowl sera.	Virus strains.				
	Cowpox.	Vaccinia.	Ectromelia.	Variola H.	Alastrim J.
Cowpox	**8**	12	5	4	3
Vaccinia	8	**12**	14	14	5
Ectromelia	8	25	**9**	9	12
Variola H	..	18	17	**10**	6
,, G	8	..	22	..	..
,, B	3	11	10	4	3
,, L	4	..	13	..	..
Alastrim	17	21	22	2	**1**
Control and normal	100	100	100	100	100

Allowing for variation in strength of various immune sera the results shown in Tables I and II indicate marked cross neutralization of the viruses by antisera prepared in the fowl. In different experiments and with different sera the numbers of lesions were reduced to from 1 to 25 per cent of that present on the membranes which received normal fowl-serum virus mixture. The cowpox antiserum and the antiserum to variola B appear to have a slightly higher antibody content in the sense that with most of the virus strains they tend to cause greater reduction in the number of lesions on the chorio-allantois.

The results shown in Tables I and II, which are representative of other similar experiments, give no indication that the neutralizing activity of these immune fowl sera was markedly specific for the individual strains of virus against which they were prepared; nor did tests with the other strains of variola virus, not shown in these tables, provide such evidence.

Neutralization of rabbit-propagated vaccinia and cowpox viruses by immune fowl sera.

The two strains of virus used in these experiments had been passed repeatedly in rabbit's skin over a number of years. They showed certain differences in the character of the lesions they produced (Downie, 1939*a*). For the neutralization tests shown in Table III and other similar experiments, elementary body suspensions from dermal infections in rabbits were used. Otherwise the technique was similar to that used in the test with viruses grown on the chorio-allantois.

TABLE III.—*The Neutralization of Rabbit-propagated Cowpox and Vaccinia Viruses by Immune Fowl Sera Prepared against Viruses Grown on the Chorio-allantois.*

Fowl sera.	Number of lesions developing on membranes inoculated with serum-virus mixtures. Cowpox virus.	Average.	Vaccinia virus.	Average.
Cowpox	3, 10, 25, 29, 30	19·6	0, 8, 12, 25, 38, D	16·6
Vaccinia	25, 31, 49, 65, D	42·5	5, 35, 36, 41, 48, 63	38
Ectromelia	3, 26, 28, 36, 51	28·8	29, 31, 36, 36, 57, 64	42·2
Variola H	10, 22, 26, 36, 46	28	11, 15, 23, 42, 50, 72	35·5
,, G	6, 15, 20, 44, D	21·2	4, 4, 10, 18, 18	10·8
,, B	1, 5, 10, 15, 17	9·6	11, 21, 22, 23, 29	21·2
,, L	13, 31, 45, 51, D	35	21, 27, 36, 46, 53	36·5
Alastrim J	0, 4, 10, 22, 34	14	12, 13, 15, 38, 42, 46	27·7
Normal and control	>200, >200, >200, >200, >200, >200, >200, >200, >200	>200	54, 81, 87, 198, >200, >200, >200, >200, >200, >200, >200	>200

>200 represents semiconfluent lesions.

These results indicate the same degree of neutralization of rabbit propagated viruses as was shown by the same immune sera against virus suspensions from infected chorio-allantoic membrane. Again there is no evidence of specificity for the homologous viruses.

The immune fowl sera were also tested for neutralizing activity against rabbit propagated cowpox and vaccinia viruses by intradermal inoculation of serum-virus mixtures into rabbits. The undiluted sera were mixed with tenfold dilutions of elementary body suspensions 1 hour before injection. Immune cowpox and vaccinia sera prepared in rabbits were tested at the same time. The results of these tests showed fair agreement with those obtained by chorio-allantoic inoculation shown in Table III. The neutralization tests in the rabbit skin suggested that (1) the antivaccinial fowl serum was rather weaker than the antivariolar fowl sera; (2) the anticowpox fowl serum was relatively strong and neutralized cowpox virus somewhat better than it neutralized vaccinia; (3) the anti-ectromelia fowl serum neutralized both viruses quite well, and (4) the fowl antisera were all much weaker than rabbit antivaccinial and anticowpox sera.

In the experiments which gave the results shown in Table III, immune sera prepared in rabbits against cowpox and vaccinia viruses were also tested. The

cowpox immune serum when mixed with the cowpox virus suspension reduced the number of lesions to 1, 1, 2, 7 and 9 on individual membranes. The vaccinia immune serum prepared in the rabbit completely neutralized the vaccinia virus suspension so that no lesion resulted from the inoculation of the mixture on four membranes. The results of further experiments with these two rabbit sera follow.

The neutralization in the chorio-allantois of viruses propagated in rabbits and in the chorio-allantois by immune sera prepared in rabbits.

The rabbit antisera to cowpox and vaccinia viruses were tested against egg and rabbit propagated strains to find out whether the method of propagation affected the specificity of the viruses for rabbit antisera. The results of two tests are shown in Table IV, the tests with rabbit-adapted and egg-grown viruses being made on different days. The antisera, which were known to have a high antibody content, were diluted 1 in 5 with normal rabbit serum before mixing with virus suspensions.

TABLE IV.—*Neutralization Tests Against Cowpox and Vaccinia Viruses with Antisera Prepared in the Rabbit Against Rabbit-adapted Strains.*

Rabbit sera.	Number of lesions developing on membranes inoculated with serum-virus mixtures. Virus strains propagated in rabbits only.			
	Cowpox.	Average.	Vaccinia.	Average.
Anticowpox	1, 1, 2, 2, 2, 5	2·2	1, 5, 6, 7, 17	7·2
Antivaccinial	1, 3, 3, 7, 7, 9	5	1, 1, 4, 4, 9	3·8
Normal	51, 56, 58, 112, 138	83	>200, >200, >200, >200, >200	>200
	Virus strains grown on the chorio-allantois only.			
	Cowpox.	Average.	Vaccinia.	Average.
Anticowpox	0, 6, 8, 8, 10, D	6·4	1, 1, 4, 5, 27	7·6
Antivaccinial	1, 3, 7, 12, 14, 19	9·3	0, 6, 9, 12, 14	8·2
Normal	130, 290, >200, >200, >200	>200	31, 52, 57, 59, 65	52·8

Although exact comparison of the results with egg-grown and rabbit-adapted viruses is not possible because accurate estimation of the number of lesions with the normal serum-virus mixture was not possible in all cases, the strains were neutralized by the rabbit antisera, irrespective of the source of virus. Previous experiments with cowpox and vaccinia viruses in rabbits had shown that cross neutralization with immune sera was practically complete, although minor immunological differences could be brought out by absorption of immune sera with concentrated virus suspensions (Downie, 1939*b*).

Neutralization tests on the chorio-allantois with smallpox convalescent and post-vaccination sera.

Variola virus from infected chorio-allontoic membranes is readily neutralized by smallpox convalescent sera and by sera collected two to three weeks after vaccination with calf lymph. The results of such a test are to be found in a previous paper (Downie and Dumbell, 1947). Table V shows the findings with such sera against a variola virus and Table VI a similar test against a vaccinia strain propagated on eggs.

TABLE V.—*Neutralization of Variola Virus by Smallpox Convalescent Serum and by Post-vaccination Serum.*

Serum.	Lesions on chorio-allantois.	Average.
Smallpox convalescent	6, 7, 9, 11, 12, D	9
Post-vaccination	13, 14, 20, 21, 58, 69	32·5
From unvaccinated adult	>500, >500, >500, >500, >500, >500	>500

>500 represents semiconfluent lesions.

TABLE VI.—*Neutralization of Vaccinia Virus by Smallpox Convalescent Serum and by Post-Vaccination Serum.*

Serum.	Lesions on chorio-allantois.	Average.
Smallpox convalescent	1, 3, 5, 12, 13, 14	8
Post-vaccination	2, 5, 6, 6, 7, 8	5·7
From unvaccinated adult	26, 38, 60, 87, 102, 109	70·3

The results of these two tests are not strictly comparable, as the same post-vaccination serum was not used in both. The serum used in the test shown in Table VI was collected 17 days after primary vaccination and had approximately the same complement fixing titre against a rabbit vaccinial antigen and against a smallpox crust antigen as did the smallpox convalescent serum. The post-vaccination serum used in the test shown in Table V had a much lower complement fixing titre.

These neutralization tests on the chorio-allantois, however, do show fairly complete cross neutralization of the two viruses by human antisera. Neutralization tests made by intradermal inoculation into rabbits of vaccinia virus mixed with such sera showed similar cross neutralization results.

DISCUSSION.

The results of the neutralization tests with antisera prepared in fowls have shown that these sera possess definite antibody activity, not only again the virus strains used to produce the sera but against all the viruses examined. That this result was not due to antibody to chick embryo protein associated with the immunizing virus preparations was shown by the results of testing the sera against rabbit-propagated vaccinia and cowpox viruses (Table III), and confirmed by neutralization tests made in the rabbit. The fowl sera were considerably weaker than sera prepared against vaccinia and cowpox viruses by hyperimmunization of rabbits. Although the birds were immunized by injections of living virus, the absence of symptoms or signs of infection and the failure to recover virus by repeated blood culture and examination of faeces following the immunizing injections suggested that there was no great multiplication of the virus in the tissues ; this may to some extent have accounted for the relatively low antibody content of the fowl sera.

A similar lack of specificity in neutralizing activity was shown by rabbit antisera when tested against vaccinia and cowpox viruses propagated either in chick embryo membranes or rabbit's skin (Table IV). Human sera from smallpox convalescents and recently vaccinated individuals also failed to distinguish egg-propagated variola and vaccinia viruses when tested by neutralization on egg membranes. Blattner, Heys and Gollub (1943) have used this method with

vaccinia virus for estimating the curve of antibody production following vaccination and during convalescence from smallpox. Neutralization tests by the egg technique have not in our experiments shown significant immunological differences in this group of viruses. It may be that cross absorption of sera with the various viruses might have brought out minor antigenic differences, but we have not as yet carried out such experiments.

Virus haemagglutinins and their neutralization by antisera have recently been employed in the study of this group of viruses (Burnet, 1946). North (1944) found that vaccinia virus preparations from the chorio-allantois agglutinated certain normal fowl red cells to higher titre than did corresponding preparations of variola virus. Neutralization of these haemagglutinins with human immune sera suggested that variola antisera were more potent against vaccinia virus suspensions than were vaccinia antisera against variola virus suspensions, but the differences were not marked. The haemagglutinins of ectromelia and vaccinia viruses affect the same range of red cells except that mouse cells are agglutinated by ectromelia and not by vaccinia (Burnet and Boake, 1946). Neutralization tests with immune sera from mice, rabbits and man showed that these haemagglutinins were closely related but not identical. Burnet and Boake further found that rabbits could be effectively immunized against vaccinia by inoculation with ectromelia virus and that a similar immunity against ectromelia infection could be induced in mice by previous injections of vaccinia virus. These authors comment on the obvious difference in the lesions produced by these viruses on the chorio-allantois and the absence of Guarnieri bodies in vaccinial membranes as contrasted with the prominent eosinophilic cytoplasmic inclusions in ectromelia lesions. It may be noted, however, that strains of cowpox virus derived from the " natural " disease in cows regularly produce strongly acidophilic cytoplasmic inclusions in infected chorio-allantois similar in appearance to those of ectromelia (Downie, 1939*a*).

Horgan and Haseeb (1945) observed that " vaccinia " strains derived from variola major, alastrim and cowpox, when tested by cross immunity experiments in different species of animals were immunologically identical. Their study of strains of variola major, alastrim and vaccinia in monkeys also indicated almost complete cross immunity between them (Horgan and Haseeb, 1939). They favour the view that immunogenic capacity is directly related to the amount of common basic antigen present and that the virulence of variola for man is associated with a specific antigen which disappears when variola, by adaptation to the rabbit, becomes degraded to vaccinia. This view is supported by the work of Amies (1932), who found that smallpox convalescent sera agglutinated variola elementary bodies but failed to agglutinate vaccinia virus. It is possible that the virus strains used in the study here reported may have varied by even limited passage on the chorio-allantois, notwithstanding the constant distinctive characteristics of the lesions produced by the different viruses in that tissue. This possibility is at present under investigation.

SUMMARY.

Immune sera were prepared by repeated injection into fowls of virus suspensions from infected chick chorio-allantoic membranes. Such antisera to virus strains from variola major, alastrim, vaccinia, cowpox and ectromelia were

examined for neutralizing activity by inoculating serum-virus mixtures on the chorio-allantois of 10–13-day chick embryos.

The results of these tests showed a close immunological relationship between the different strains of virus examined. Using vaccinia and cowpox viruses propagated in rabbits, these sera when tested in the same way gave similar results. These findings were also confirmed by tests with the various fowl sera made by intradermal inoculation of serum-virus mixtures into rabbits. Immune sera prepared in rabbits against cowpox and vaccinia viruses, and human antisera against variola and vaccinia viruses also failed to bring out specific antigenic differences by neutralization tests made in the chorio-allantois.

The results have been discussed in relation to the findings of other workers using different methods and the possible influence of variation of the virus strains by propagation on the chorio-allantois has been considered.

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TRANSPORTATION OF THE ANAPHYLACTOGENIC PROPERTY BY EOSINOPHIL.

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THE origin, nature and rôle of the eosinophils in physiological and pathological conditions are still obscure ; many observations of their behaviour have been recorded, but most of them are rather indefinite. The absolute eosinophil-count fluctuates even in physiological conditions, e.g. after long sleep at night the blood eosinophilia is higher than during the day (Domarus, 1931) ; also adrenaline and drugs stimulating adrenaline release produce eosinopenia lasting several hours (Godlowski, 1948). It is a well established and generally accepted fact that in allergic conditions hypereosinophilia appears in the blood or in the tissues of the affected organs or in both, and this is the most constant and characteristic feature of these conditions (Ringeon, 1938). On the other hand, eosinophilia varies in its intensity in the course of these conditions and the relation of this fluctuation to the stages of the disease is not clear.

The constancy of the local and general eosinophilia in allergic conditions suggest that eosinophils may play a substantial rôle in the antigen-antibody interaction which is, at present, generally accepted as a causal factor of anaphylactic shock. Sabin (1939) made observations which suggest that leucocytes may transport protein, but ascribed this function to all white cells of polymorph type, including eosinophils ; she said that they phagocyted protein particles which were coupled with azo-benzidine dye and brought and deposited them in the reticulo-endothelial system ; these results are discussed below. Weinberg and Seguin (1914, 1915) claimed that eosinophils possess antigenic power. Olsen (quoted by Ringeon, 1938) indicated in his experiments on eosinophils that a specific sensitizing product is formed in the reaction between eosinophil granules and complex protein.

The object of the following experiments was to ascertain whether eosinophils are able to carry antigens and whether they are the only element among white cells of the blood possessing this property.

METHODS.

(1) *The dosage of the antigens.*

The dosage of the antigens (eosinophilic, leucocytic and sera) was calculated from the amount of Total Protein Nitrogen (T.P.N.) which was estimated by the micro-Kjeldahl method. The eosinophilic or leucocytic antigens added to the bath in the Schultz-Dale test (S.D.T.) with the specifically sensitized uterus contained approximately the same amount of T.P.N. as the shock dose of the specific antigen itself used in the S.D.T. The T.P.N. values of the eosinophilic and

leucocytic antigens obviously originated not only from the anaphylactogenic protein but also, and probably mostly, from the protein of the cytoplasm and nuclei of all cells. This may be the reason why the contractions of the uteri in S.D.T.s varied sometimes so widely in intensity.

It was found that the susceptibility of the sensitized and non-sensitized uteri to the toxic and specific antigenic action of the serum varied so widely that before each series of experiements it was necessary to estimate the standard dilution of the serum used as the shock dose. One day before actual experiments were made a control was done on the non-sensitized and sensitized guinea-pigs in order to determine (*a*) the toxic action of the serum and (*b*) the degree of uterine susceptibility to the specific antigen of the sensitized animals. The standardization was made on few animals taken at random from animals to be used for the following day's experiment and the S.D.T. was accepted as the control test.

(*a*) The toxic action of the serum was first checked on non-sensitized guinea-pigs ; the highest dilution of the serum which did not precipitate a contraction of the non-sensitized uterus, but was able to produce a contraction of the sensitized uterus, was accepted as a *preliminary standard dilution of the serum.*

(*b*) The degree of the uterine susceptibility of the sensitized animals was determined in the following way : the preliminary standard dilution sometimes produced in highly susceptible uteri a tetanic contraction, which could be avoided by further dilution of the serum. The dilution which precipitated a total contraction without tetanic period was accepted as *standard dilution* or *shock dose* for the following day's experiments. The criterion of the degree of susceptibility of the uterus in the actual experiment was the altitude of the spontaneous rhythmic contraction of the uterus after 30 minutes of preliminary recording. The uteri with the contractions about 0·5 cm. of altitude were regarded as being of higher susceptibility. By these precautions experimental error was substantially minimized but by no means eliminated.

In the first three series of experiments the standard dilution of human serum (h.s.) used was 1/1000. In the next two series the animals were divided into two categories : (*a*) Those of lower susceptibility in which the standard dilution of the human serum (hu.s.) was 1/10,000 ; and (*b*) those of higher susceptibility in which the standard dilution of the hu.s. was 1/100,000.

(2) *Production of the eosinophilic antigen obtained from guinea-pigs sensitized to horse serum (h.s.).*

Twenty selected normal guinea-pigs with an eosinophilia in the peripheral blood ranging from 11 to 29 per 100 were injected intraperitoneally with 3 ml. of h.s. 4 times, with intervals of 5 days between each injection. Twenty-four hours after the last injection the animals were killed by striking them on the head, and bled from the jugular vein. The skin of the abdomen was then cut and before the peritoneum was opened 10 ml. of sterile normal saline at 37° C. were injected into the peritoneal cavity. By means of slight massage of the abdomen the cellular elements were washed out from the peritoneal surface ; then the peritoneal cavity was aseptically opened and the whole fluid content was removed (Weinberg and Seguin, 1914, 1915). The whole collected fluid was then centrifuged and the remaining sediment was washed with normal saline and centrifuged several times until the supernatant fluid gave a negative test for albumen with

sulpho-salicylic acid. The cells from the sediment were then suspended in Locke's solution and the quantitative count of the eosinophils was made in the counting chamber, using the Randolph method (1944). The total cell-count of cellular suspension was 22,000 in 1 c.mm., of which 60 per 100 cells were eosinophils, i.e. in 1 c.mm. there were 13,000 eosinophils. Eosinophils were regarded as including the eosinophilic polymorphs and their young forms, and those which are described by some authors as pseudo-eosinophils, i.e. those mononuclear cells which contain eosinophilic granules in their cytoplasm (Ringeon, 1938).

The T.P.N. value for eosinophilic antigen was 33 mg. per 100 ml. and for the h.s. used in the S.D.T. was 1092 mg. per 100 ml., i.e. 300 times higher than for the eosinophilic antigen.

After the count of cells was made the suspension of cells was cooled at — 4° C. (which does not destroy the antigenic property of protein) and warmed up again to 37° C. ; this procedure was repeated several times in order to destroy the eosinophils and to release from their cytoplasm the protein amongst which there might be found anaphylactogenic protein.

Such a suspension of cells is called, for convenience, *eosinophilic antigen*, although it consisted of other white cells as well as eosinophils.

(3) *Production of the eosinophilic antigen obtained from guinea-pigs sensitized to egg-white albumen* (*e.w.*).

Hen's egg-white (Dienes and Schoenhiet, 1927) aseptically obtained, was diluted with an equal amount of sterile water, gently shaken and centrifuged (Campbell, Drennan and Rettie, 1935) for 30 minutes at a speed of 4000 r.p.m. ; the clear supernatant fluid was used for the production of anaphylactic peritonitis in 10 guinea-pigs in the way described in the previous paragraph ; all other details concerning T.P.N. and preparation of eosinophilic antigen from the peritoneal exudate were also carried out as described above.

Leucocytosis in the e.w. eosinophilic suspension was 11,000 in 1 c.mm., of which 26 per 100 cells were eosinophils, i.e. in 1 c.mm. there were about 2750 eosinophils. The T.P.N. for this eosinophilic antigen was 30 mg. per 100 ml. Although the T.P.N. of this antigen was approximately the same as in h.s. eosinophilic antigen, yet the eosinophilia was approximately of 1/4 of its value, therefore the e.w.-eosinophilic antigen was added to the bath in the S.D.T. in 4 times stronger concentration than h.s. eosinophilic antigen.

(4) *Production of the eosinophilic antigen from guinea-pigs sensitized to two antigens.*

Ten normal guinea-pigs with originally high blood eosinophilia were injected simultaneously with 3 ml. hu.s. intramuscularly and 3 ml. h.s. intraperitoneally on four occasions, an interval of five days being allowed between injections. Twenty-four hours after the last injection all animals were killed as described above. Cells from the peritoneal cavity were obtained and treated as described in paragraph 2. The number of cells in this series was 460,000 in 1 c.m., of which 20 per 100 cells eosinophils, i.e. 92,000 eosinophils in 1 c.mm. The T.P.N. of this eosinophilic antigen was 111 mg. per 100 ml. ; the hu.s. used for the sensitization and for S.D.T. contained 1078 mg. per 100 ml., i.e. approximately ten times more than the eosinophilic antigen.

(5) *Production of leucocytic antigen without eosinophils obtained from guinea-pigs sensitized to two different antigens.*

Ten normal guinea-pigs originally with very few or no eosinophils in the peripheral blood were selected for this experiment. In order to find out whether these animals with low eosinophilia were of poor immune function, two guinea-pigs (other than those ten) were sensitized to hu.s. by subcutaneous injection of 0·5 ml. of hu.s. and in 3 weeks' time reinjected intravenously with 0·3 ml. of hu.s.; both animals died in typical anaphylactic shock. Injection of hu.s. and h.s. and other details of the technique used for the preparation of the cell-suspension were as described above, the only modification being the intraperitoneal injection 12 hours before death of 0·37 ml./kg. of a sterile saturated solution of ammonium chloride (Josey and Lawrence, 1932). In the anaphylactic peritoneal exudate produced in such a way there were no eosinophils at all and the cells were either of the mononuclear type or neutrophil polymorphs, in more or less equal numbers. The count was made in a chamber and a differential count also was made from a film stained by Leishman. The cell-count of this suspension was 29,400 in 1 c.mm.; the T.P.N. was 38 mg. per 100 c.c., i.e. the T.P.N. value of the eosinophilic antigen from the animals sensitized to two antigens was approximately 3 times higher and that of hu.s. itself used for sensitization 30 times higher.

(6) *Schultz-Dale Test* (*S.D.T.*) (Schultz, 1910, 1911; Dale, 1912–13).

The test was made on the uteri of virgin guinea-pigs weighing less than 250 g.; three weeks before the experiments they were sensitized by intraperitoneal injection of 1 c.c. of (*a*) undiluted h.s.; (*b*) undiluted hu.s.; (*c*) eosinophilic antigen obtained from h.s. peritonitis (see paragraph 2).

H.s. and hu.s. were obtained from the venous blood of normal animals and human-beings; the sera used for the S.D.T. were at least a fortnight old.

The animals were killed in the way previously described immediately before each experiment and the two horns of the same uterus were placed in two separate containers, each of 10 c.c., which were immersed in one bath at a temperature of 38° C. The Locke's solution used in the S.D.T. was made according to Dale's formula (Dale and Laidlaw, 1912–13) and prepared immediately before the experiments. After each contact of the muscle with antigen and before the introduction of another dose of the same or different antigen the solution of the bath was changed several times.

The activities of the antigens were checked on two horns of the same uterus sensitized to the antigen, which was supposed to be carried by the eosinophils, by the following method: (*a*) One horn received the shock dose of the original antigen by which the animal was sensitized, producing a contraction followed by refractoriness of the muscle to a second dose of the same antigen and to the antigen to be tested, i.e. eosinophilic antigen; (*b*) the second horn of the same uterus served for the experiment conducted in a reversed order, i.e. the antigen to be tested was given first, and this resulted in a contraction, followed by refractoriness to a second dose of the same antigen and to the original antigen—if the experiments were positive. At the end of each experiment 1μg. of histamine base (Halpern and Dainow, 1943) was added to the bath in order to prove that the muscle could still contract; if, however, spontaneous contractions occurred this procedure was omitted.

RESULTS.

The approach to the problem of antigen transportation by eosinophils was made through five separate experiments.

Experiment 1 was carried out on four h.s.-sensitized and on three normal (control) guinea-pigs. To the bath with one horn of the uterus sensitized by h.s. there was added a shock dose of h.s. 1/1000 dilution, at the first arrow of Fig. 1, A, which produced a strong contraction. The second shock dose of h.s. added at the second arrow found the muscle completely refractory to it and this refractoriness also persisted on the addition of the shock dose of the h.s.-eosinophilic antigen in 1/300 dilution (at the third arrow). The second horn of the same uterus (Fig. 1, B) when exposed to the shock dose of the h.s.-eosinophilic

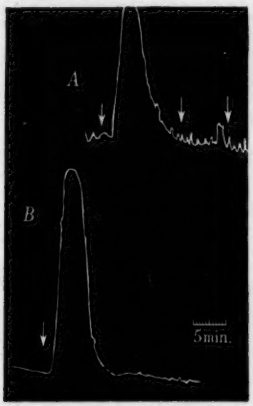

FIG. 1.—Guinea-pig's uterus horse serum sensitive. A and B are two horns of the same uterus.
A. First arrow : horse serum 1/1000 ; second arrow : same dose repeated ; third arrow: horse serum-eosinophilic antigen 1/300.
B. Horse serum-eosinophilic antigen 1/300.
Concentration of antigens figures refer to the actual concentrations in bath.

antigen in 1/300 dilution, produced a vigorous contraction. In another case of the same series (Fig. 2, A) the first shock dose of the h.s. in 1/1000 dilution (at the first arrow) after a contraction, produced refractoriness of the muscle to the second shock dose of the h.s. (at the second arrow) and to the shock dose of h.s.-eosinophilic antigen (third arrow), but the uterus retained a high susceptibility at this stage towards 1μg. of histamine base (fourth arrow). The second horn of the same uterus (Fig. 2, B), however, to produce refractoriness needed a contact with two successive shock doses of the h.s.-eosinophilic antigen (first and second arrows) and then remained refractory also towards a shock dose of the h.s. itself, added to the bath at the fourth arrow ; at this stage again the muscle showed a high susceptibility towards 1 μg. of histamine base.

The uteri of the normal animals remained unaffected by the shock dose of the h.s. and of the h.s.-eosinophilic antigen, but responded well to 1 μg. of histamine base.

Experiment 2 was carried out on the uteri of five sensitized and three normal guinea-pigs. To the bath containing one horn of the h.s. sensitive uterus there was added at the first arrow of Fig. 3, A, a shock dose of the e.w.-eosinophilic

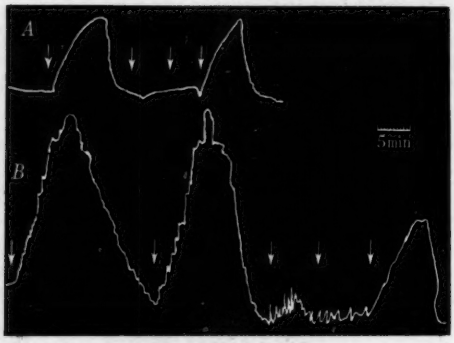

FIG. 2.—Experimental conditions as in Fig. 1.
A. First and second arrows: horse serum 1/1000; third arrow: horse serum-eosinophilic antigen 1/300; fourth arrow: 1 μg. of histamine base.
B. First, second and third arrows: horse serum-eosinophilic antigen 1/300; fourth arrow: horse serum 1/1000; fifth arrow: 1 μg. histamine base.

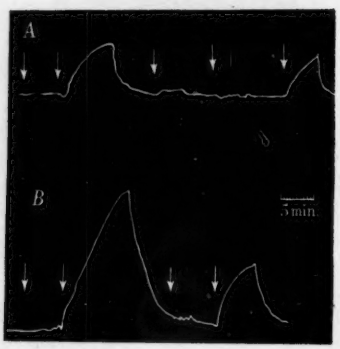

FIG. 3.—Experimental conditions as in Fig. 1.
A. First arrow: egg-white-eosinophilic antigen 1/75; second and third arrows: horse serum-eosinophilic antigen 1/300; fourth arrow: horse serum 1/1000; fifth arrow: 1 μg. histamine base.
B. First arrow: egg-white-eosinophilic antigen 1/75; second and third arrows: horse serum 1/1000; fourth arrow: 1 μg. histamine base.

antigen in 1/75 dilution, which produced no visible effect on the muscle, whereas the contact made at the second arrow with the shock dose of h.s.-eosinophilic antigen in 1/300 dilution produced a contraction; the second shock dose of the h.s.-eosinophilic antigen found the muscle refractory to it (at the third arrow) and the refractoriness remained also towards the shock dose of the h.s. itself added at the fourth arrow; at this stage, however, 1 μg. of histamine base (at

the fifth arrow) precipitated a marked contraction. The second horn of the same uterus remained also unaffected by the shock dose of the e.w.-eosinophilic antigen in 1/75 dilution (at the first arrow of Fig. 3, B), but responded with a contraction to the shock dose of h.s. in 1/1000 dilution. The second shock dose of h.s. (at the third arrow) found the muscle refractory to it, but the muscle was highly susceptible to 1 μg. of histamine base added at the fourth arrow. Experiments

FIG. 4.—Control guinea-pig, otherwise conditions as in Fig. 1.
A. First arrow: egg-white-eosinophilic antigen 1/75; second arrow: horse serum-eosinophilic antigen 1/300; third arrow: 1 μg. histamine base.
B. First arrow: egg-white-eosinophilic antigen 1/75; second arrow: horse serum 1/1000; third arrow: 1 μg. histamine base.

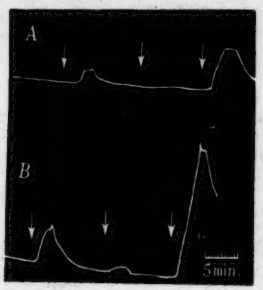

FIG. 5.—Guinea-pig's uterus sensitized by horse serum-eosinophilic antigen otherwise as in Fig. 1.
A and B. First and second arrows: horse serum 1/1000; third arrow: 1 μg. histamine base.

conducted in a similar way on four other guinea-pigs gave approximately the same result, but in three of these animals the single shock dose of h.s.-eosinophilic antigen did not desensitize the uterus against the shock dose of h.s.

The uteri of all control animals, one of which is presented in Fig. 4, A and B, remained unaffected when exposed to shock doses of the e.w.-eosinophilic antigen, that of h.s.-eosinophilic antigen and that of the h.s. itself, but all of them responded with contractions when acted upon by 1 μg. of histimane base.

Experiment 3 was carried out on four guinea-pigs sensitized by h.s.-eosinophilic antigen and on three controls. Sensitization of guinea-pigs was made by

intraperitoneal injection of 1 ml. of h.s.-eosinophilic antigen 3 weeks before the actual experiment took place; the details of the h.s.-eosinophilic antigen preparation were described in paragraph 2. The shock doses of h.s. in 1/1000 dilution were added to the two separate baths containing the horns of the same uterus at the first arrows of Fig. 5, A and B, and precipitated a contraction of the muscle followed by refractoriness to the second doses of h.s. added at the second arrows. Both horns at this stage of refractoriness produced the usual contraction when acted upon by 1 μg. of histamine base (third arrow). The uteri of the other three sensitized animals responded to the shock dose of h.s. approximately in the same manner as described above; whereas three control animals remained unaffected when exposed to shock dose of the h.s., but produced a contraction when acted upon by 1 μg. of histamine base.

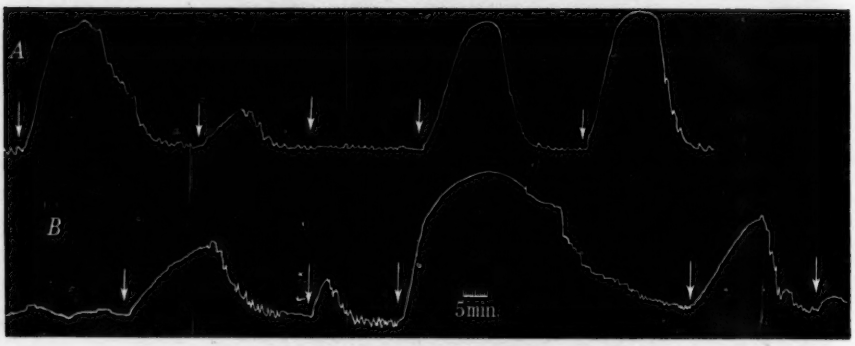

FIG. 6.—Guinea-pig's uterus sensitized by human serum, otherwise as in Fig. 1.
A. First and second arrows: human serum 1/10,000; third arrow: eosinophilic antigen 1/1000; fourth and fifth arrows: 1 μg. histamine base.
B. First and second arrows: eosinophilic antigen 1/1000; third and fourth arrows: human serum 1/10,000; fifth arrow: eosinophilic antigen 1/1000.

Experiment 4 was made on four hu.s. sensitized and on two control guinea-pigs. To the bath with one horn of the hu.s. sensitive uterus a shock dose of the hu.s. in 1/10,000 dilution was added (first arrow of Fig. 6, A) and produced a contraction and subsequent partial refractoriness to the second shock dose of the hu.s. (at second arrow) and also to the shock dose of the eosinophilic antigen in 1/1000 dilution (at the third arrow), obtained from h.s. peritonitis from guinea-pigs (which had been simultaneously injected with hu.s.). The muscle remained, however, fully susceptible at this stage to two successive doses of 1 μg. of histamine base (at the fourth and fifth arrows of Fig. 6, A). In tracing B, at the first arrow the shock dose of the hu.s.-eosinophilic antigen precipitated a definite contraction of the second horn of the same uterus; the second dose of the same antigen added to the bath at the second arrow resulted only in a toxic contraction, but both these contractions were unable to desensitize the muscle to a shock dose of the hu.s. itself in 1/10,000 dilution when the muscle was exposed to it at the third arrow of the tracing B in Fig. 6; the second dose of the hu.s. produced a much weaker contraction and both these contractions made the muscle refrac-

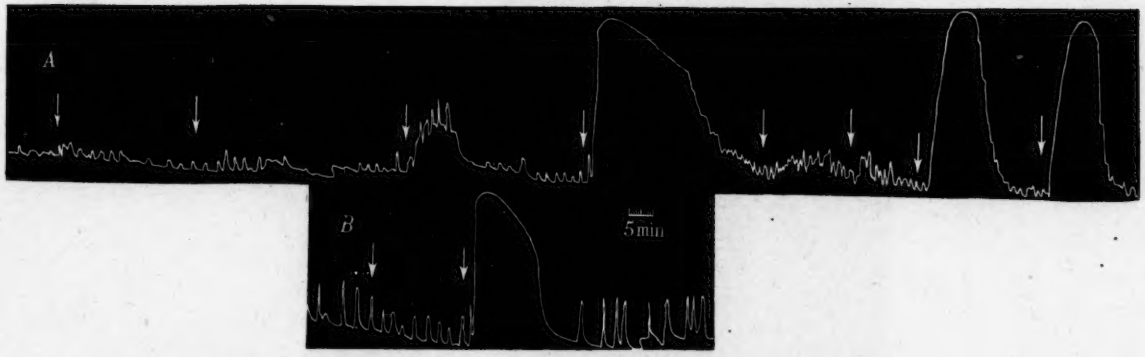

FIG. 7.—Experimental conditions as in Fig. 6.

A. First and second arrows: leucocytes antigen without eosinophils in 1/300 and 1/30; third arrow: eosinophilic antigen 1/1000; fourth and fifth arrows: human serum 1/100,000; sixth arrow: eosinophilic antigen 1/1000; seventh and eighth arrows: 1 μg. histamine base.

B. First arrow: leucocytes antigen without eosinophils 1/300; second arrow: human serum 1/100,000.

tory to the shock dose of the hu.s.-eosionophilic antigen in 1/1000 dilution added to the bath at the fifth arrow. The other three sensitized guinea-pigs responded to the experiment in approximately the same manner ; in one of them, however, a single shock dose of the hu.s.-eosinophilic antigen was capable of producing refractoriness towards a second dose of the same antigen as well as towards the shock dose of hu.s. itself.

The uteri of the two control animals showed no response at all when acted upon by the shock dose of the hu.s. in 1/10,000 dilution and by the shock dose of the hu.s.-eosinophilic antigen. Both horns contracted in the usual manner on contact with 1 μg. of histamine base.

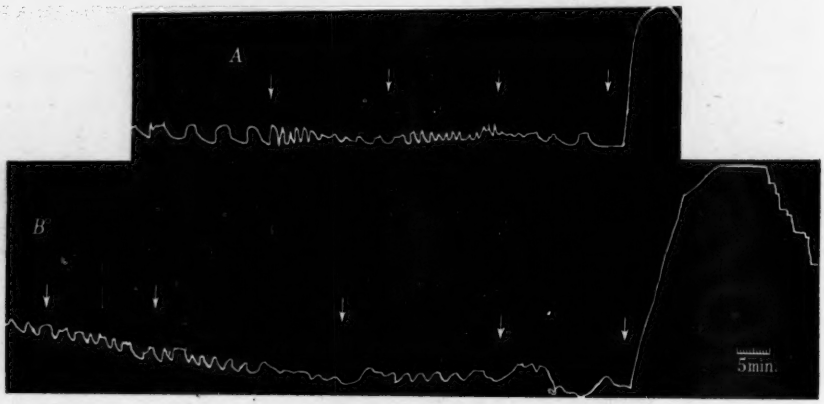

FIG. 8.—Control guinea-pig, otherwise as in Fig. 6.
A. First arrow: leucocytes antigen without eosinophils 1/300; second arrow: eosinophilic antigen 1/1000; third arrow: human serum 1/100,000; fourth arrow: 1 μg. histamine base.
B. First and second arrows: eosinophilic antigen 1/1000 and 1/100; third arrow: leucocytes antigen without eosinophils 1/30; fourth arrow: human serum 1/100,000; fifth arrow: 1 μg. histamine base.

Experiment 5 was carried out on one control and two hu.s.-sensitized guinea-pigs. In the bath containing one horn of the hu.s.-sensitive uterus the shock dose of the leucocyte antigen without eosinophils in 1/300 dilution was in contact with the muscle at the first arrow of Fig. 7, A, and did not affect the muscle in any visible way. At the second arrow the uterus was exposed to a ten times stronger dose of the same antigen, i.e. in 1/30 dilution, but this also did not produce any effect on the muscle. At the third arrow the uterus was exposed to the shock dose of the hu.s. eosinophilic antigen in 1/1000 dilution, which precipitated a contraction, but it was not able to desensitize the muscle against the shock dose of the hu.s. itself (at the fourth arrow). The contraction precipitated by hu.s. produced then a complete refractoriness of the muscle to the shock dose of the hu.s. itself (fifth arrow) and that of hu.s.-eosinophilic antigen (sixth arrow), but the muscle remained at this stage fully susceptible to the two successive doses of 1 μg. of histamine base (at seventh and eighth arrows of Fig. 7, A).

The second horn of the same uterus (Fig. 7, B) exposed to the shock dose of the leucocytic antigen without eosinophils in 1/300 dilution produced no visible reaction. The sequence of reactions in an experiment carried in similar way on another sensitized guinea-pig confirmed the above described results.

Neither horn of the uterus of the control animals responded when acted upon by the shock dose of the leucocytic antigen, or hu.s.-eosinophilic antigen and hu.s. itself, but strongly contracted when it received a dose of 1 μg. of histamine base (Fig. 8, A and B).

DISCUSSION.

Guinea-pig uteri sensitized to the h.s. responded with a contraction when they received a shock dose of the h.s. or of h.s.-eosinophilic antigen. This is clearly shown in the first experiment. In other words the h.s.-eosinophilic antigen possesses the property of the specific serum, provided that any non-specific factor in the eosinophilic antigen can be excluded. It has been already mentioned in the section on methods that T.P.N. was accepted as the basis for the dosage of antigens. On the other hand the T.P.N. value was stated to represent all the proteins which the eosinophilic suspension originally possessed and it seems reasonable to accept that the greater part of the protein which T.P.N. represents originates from the cytoplasm and nuclei of the cells themselves. Naturally, the anaphylactogenic protein might and does fluctuate in its amount, therefore the dose of the eosinophilic antigen based on the value of T.P.N. will also fluctuate and thus the dosage of antigen is only very approximate. This may be the reason why uterine contractions precipitated by the so-called " shock dose " of the eosinophilic antigen did not produce a complete shock of the muscle and subsequent complete refractoriness. Although the contraction itself may be very striking, as, for example, in Fig. 2, B, yet it might result from summated stimuli of non-specific and specific origin, and the specific component may be of inadequate strength to produce shock and complete desensitization of the muscle.

As to the question of the specificity of the oesinophilic antigen, the second experiment brings sufficient evidence against the influence of the non-specific factor in the action of this antigen. Recently published reports attribute the contraction of smooth muscles during anaphylactic shock to the release of histamine in this shock (Schild, 1939 ; Dragstedt, 1941 ; Rose, Feinberg, Friedleander and Feinberg, 1947 ; Gaddum, 1948). On the other hand, there are reports (Code, 1937) claiming that amongst white cells of the blood eosinophils are the richest source of histamine. If the anaphylactic contractions were entirely due to the histamine present in the eosinophils, so all eosinophilic antigens, regardless of their origin, should equally well precipitate a contraction of h.s.-sensitized and non-sensitized uterus, despite the fact that they never made any contact with the specific antigen. In the second experiment h.s.-sensitized uterus was treated by the eosinophilic antigen obtained from the peritoneal exudate produced by the repeated injection of egg-white albumen ; the uterus, however, remained entirely unaffected by this eosinophilic suspension, although the amount of T.P.N. and the number of eosinophils were calculated in accordance with those of shock dose of the h.s.-eosinophilic antigen. Thus this experiment excludes the possibility that the h.s.-eosinophilic antigens' action on a uterus sensitized by h.s. is non-specific.

The third experiment only confirmed the results obtained in the first two experiments. If the factor in the eosinophilic antigen producing contraction of

a sensitized uterus was non-specific, such antigen could not itself sensitize the normal uterus towards h.s. The relatively weak contraction of the uterus sensitized by means of h.s.-eosinophilic antigen when exposed to h.s. (Fig. 5, A and B) results from the inadequate dose of the anaphylactogenic protein in the eosinophilic antigen used for the sensitization of the animal.

In all three experiments just discussed the cells used as antigen were in local contact with the h.s. itself in the peritoneal cavity from which the cells came and into which the h.s. was repeatedly injected. One cannot neglect the possibility, therefore, that the specific property of the cells' suspension may be due to the local adhesion of h.s. to the surface of these cells. There may be, however, another explanation of this phenomenon, namely, the cells may phagocyte the protein in the same way as they do other foreign colloid which contacts them. In order to eliminate possible adhesion, four animals were sensitized to two different antigens injected into two anatomically separate places. One of them (h.s.) was repeatedly injected intraperitoneally to produce anaphylactic peritoneal exudate for the collection of eosinophils, and the other antigen (hu.s.) intramuscularly. Even after taking this precaution there may be still a chance that the hu.s. injected intramuscularly, circulating in the guinea-pig's organism, may be excreted into the anaphylactically inflamed peritoneal cavity. In such circumstances there is a possibility of local contact between the cells of the peritoneal exudate and hu.s. The contact between the white cells and specific antigen may also take place in the blood stream and eosinophils loaded with anaphylactogenic protein may subsequently gather in the peritoneal cavity. Wherever this contact occurs it *is essentially important that eosinophils make a local contact with the specific antigen, if they are to acquire the antigenic property, in this case the specific property of hu.s.*

As Fig. 6, B, shows, the shock of the hu.s.-eosinophilic antigen produced a fairly strong contraction of the uterus sensitized to hu.s.; the second dose of the same antigen produced a much weaker contraction; both these doses, however, were unable to desensitize the muscle against the shock dose of the hu.s. itself. The second horn of the same uterus (Fig. 6, A) desensitized by the shock dose of h.s. remained also refractive to the shock dose of hu.s.-eosinophilic antigen, but at this stage of refractivity the muscle remained highly susceptible to the two successive doses of 1 μg. of histamine base.

The conclusions from this experiment may be summarized as follows: the contraction of the uterus sensitized by hu.s., when treated with eosinophilic antigen—which was never in close contact with hu.s. itself, but was obtained from the peritoneum of animals which were injected intramuscularly with hu.s.—was precipitated by a specific agent carried by eosinophils, this contact resulted in an anaphylactic shock which took place in the muscle. The refractivity of the uterus to the repeated shock dose of hu.s. or of eosinophilic antigen was due to the exhaustion of the biochemical reactions which occur during anaphylactic shock; if the dose of the anaphylactogenic protein in the antigen is not adequate for the production of maximal shock, the desensitization of the muscle is not complete. The desensitized uterus resulting from maximal anaphylactic shock caused by the hu.s. or by eosinophilic antigen remains highly susceptible to the non-specific action of 1 μg. of histamine base.

If hu.s. injected intramuscularly was really excreted into the peritoneal cavity and local contact with the cellular elements of the peritoneal exudate took place,

then all cells would have the same chance to pick up or adsorb hu.s. and all of them should be able to exercise the same specific action as eosinophils did upon the uterus sensitized by hu.s. Fig. 7, A, represents one horn of the uterus sensitized by hu.s. which was exposed twice to a shock dose of the leucocytic antigen without eosinophils obtained from guinea-pigs injected with two antigens (in the same manner as animals from the fourth experiment). Neither of these doses exercised any visible effect on the uterus, whereas eosinophilic antigen obtained in a similar way caused a contraction, which, however, did not desensitize the muscle against the shock dose of the hu.s. itself.

The conclusions to be drawn from these experiments are: white cells other than eosinophils have not the property of acquiring a specific antigenic action if obtained from the peritoneal cavity of animals which were injected intramuscularly with specific antigen. If local contact of the cells with hu.s. took place in the peritoneal cavity, the eosinophils are the cells which are able to become the carriers of the specific antigen.

The question now arises whether the acquisition of the antigenic property by eosinophils is purely incidental or whether the eosinophils are predestined to this function. The experiments made by Weinberg and Seguin (1915) showed that eosinophils possessed antigenic power in the haemolysis in the presence of complement. Sabin (1939) showed in experiments with dyed protein that leucocytes (including eosinophils) were the cells which transport the protein to the reticulo-endothelial system, in which they were eventually phagocyted together with the coloured protein. These experiments proved only the fact that the colloidal dye coupled with protein was phagocyted by leucocytes and deposited in the reticulo-endothelial system. This, incidentally, is also the mechanism of the phagocytosis of all colloidal dyes in so called colloidal blockade of the reticulo-endothelial system. From Sabin's experiments it is impossible to say whether the dyed protein has been phagocyted by this mechanism because colloidal dye was included in the dyed-protein particles and the protein itself was a compulsory fellow-traveller, or whether protein itself without dye travels the same way.

On the basis of the results presented here one has to assume that one rôle of eosinophils is transportation of antigen; these experiments, however, cannot be accepted as conclusively proving that this function is the main or the only function of the eosinophils. Such a view may, however, find some support in the well known fact that in all anaphylactic alterations in the tissues the local eosinophilia is one of the most constant and most characteristic features. If the theory suggested above is correct the eosinophils may transport the antigen to the site of interaction between antigen and antibody, which according to the generally accepted view is the primary cause of the anaphylactic shock.

SUMMARY.

1. Antigenic extracts of eosinophils from the anaphylactic peritoneal exudate of guinea-pigs were tested for the presence of the specific anaphylactogenic protein by means of specifically sensitized uterus muscle.

2. The anaphylactogenic protein is present in the eosinophil cells and could not be detected in other white cells.

3. A theory of the rôle of eosinophils in allergic processes is suggested.

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ELECTROPHORETIC STUDIES ON THE SERUM OF GOLDEN HAMSTERS INFECTED WITH *LEISHMANIA DONOVANI*.

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THE progress of human kala-azar is accompanied by an absolute decrease in serum albumin and a considerable rise in total serum globulin. Most and Lavietes (1947), in electrophoretic studies on the serum of kala-azar patients, found that the increase occurred in the γ-globulin fraction and that there was none in the α- and β-fractions. Cooper, Rein and Beard (1946) noted abnormalities in the γ-globulin component in the serum of two kala-azar patients and concluded that "The electrophoretic abnormalities may prove to be unique with kala-azar and of greater diagnostic value than the formol gel test or cold precipitation." Since we were engaged on studies of experimental leishmaniasis of hamsters infected with an Indian strain of *L. donovani*, it appeared to us of interest to investigate the electrophoretic pattern of the serum during the complete cycle of this fatal

disease. In the hamster infection, Stein and Wertheimer (1942) found, by chemical methods, that there was no change in total protein of the serum up to 46 days after inoculation, and that abnormal proteins were absent ; but Gellhorn, van Dyke, Pyles and Tupikova (1946) found, also by chemical methods, a significant decrease in plasma albumin from the 46th day after inoculation onwards without alteration in the globulin concentration. There was accompanying nephritis, proteinuria, oedema, amyloidosis of various organs and a decrease of plasma proteins.

Our observations on untreated animals were made at intervals over a period of 90 days from the time of inoculation. When the infection became severe deaths were frequent and the pathological lesions described above were observed. Treatment of some infected animals with an organic antimonial caused a temporary halt in the progress of the infection and allowed examination of their serum to be made up to 105 days after infection. The pooled serum of control animals was examined at similar intervals. Serum from two human patients with kala-azar was also included in these examinations.

MATERIAL AND METHODS.

Biological.

A batch of 150 golden hamsters of both sexes, aged 5–10 weeks, were used. One hundred were inoculated intraperitoneally with $\frac{1}{2}$ c.c. suspensions in Ringer-glucose, of pulped spleen from infected hamsters, which had been passed through a fine layer of cotton wool to remove grosser tissue particles. Each inoculum contained approximately 56 million Leishman-Donovan bodies. Batches of infected animals were killed at intervals during the course of the disease and their pooled serum, as well as that of uninfected controls, was examined electrophoretically. In order to observe if any changes were produced in serum as the result of injection of splenic tissue, ten animals were injected with a suspension of uninfected hamster spleen and their sera examined 59 days later, at a time when marked changes were apparent in those of infected animals. When the leishmanial infection, was well developed 20 hamsters were given a single intraperitoneal dose of 800 mg. sodium stibogluconate per kilogram and their pooled serum was examined at intervals of 12, 25 and 40 days after treatment. Five normal hamsters were given the same dose of drug and their serum was examined 23 days later to find whether any change had been produced by the treatment. The degree of splenic infection of all animals was determined in stained smears by counting the number of Leishman-Donovan forms per field with the aid of a 1/12 in. oil immersion lens and 6× eyepiece. At the earliest examinations, when parasites were hard to find, cultures of spleen were made on Locke-blood-agar medium to establish the presence of leishmanial infection. Rectal temperatures and the weight of the spleen at necropsy were recorded throughout the course of the disease.

Electrophoretic.

The sera were diluted with an equal volume of potassium phosphate buffer pH 8 and ionic strength $\mu 0{\cdot}2$. After dialysis for 24 hours at 2° C. against two changes of buffer, they were further diluted and the process of dialysis was continued for the same period. The final refractive increment in the protein solutions

was 0·003. An electrophoretic cell of 11 c.c. capacity was employed with a current of 15 milliamps over a period of 6 hours. As the disease progressed a lipaemia developed which made the serum, even after considerable dilution, too turbid for electrophoretic work. On that account three extractions with ether were carried out as described by McFarlane (1942). In the later stages of the infection only one extraction was possible owing to the reduced stability of serum protein. Isolation of phospholipids was carried out by the method of Bloor (1929) and they were estimated by the method of Allen (1940). Cholesterol

TABLE I.—*Details of Leishmanial Infections in Hamsters.*

	Day of infection.	Number of animals examined.	Mean splenic weight. mg.	Parasites in smears of infected spleen.
Infected animals untreated	10	6	172	+
	21	7	204	++
	28	8	211	+ to ++
	38	8	213	+++ to ++++
	50	7	363	++++ to +++++
	63	11	585	++ to +++++
	77	8	436	+++++
	90	10	371	++++ to +++++
Infected animals treated with antimony*	77	7	208	None.
	90	7	263	+
	105	3	280	+ to ++
Uninfected controls	0	7	67	—
	50	8	69	—
	77	5	148	—
	90	6	166	—
	105	9	127	—

* Antimony treatment was given on the 65th day of infection.

\+ = spleen smear scanty and culture positive.
++ = 1 to 100 L.D. forms per 100 microscopic fields.
+++ = 101 „ 1000 „ „ „ „ „
++++ = 1001 „ 5000 „ „ „ „ „
+++++ = >5000 „ „ „ „ „

was estimated by the method of Sperry and Brand (1943) and fatty acid, excluding that of phospholipid fatty acid, by that of Bloor (1928).

RESULTS.

From the data in Table I it will be seen that, in most animals, the infections were of the usual rapidly progressive character as judged by the increase in the number of parasites in smears of the spleen and by increase in size of that organ. Treatment with antimony caused a temporary marked reduction in the number of parasites without eradicating the infection and the number of parasites soon began to increase again. The temperature of infected animals which was approximately 100° F. showed no significant alterations from that of controls throughout the course of the disease. The formol gel test was consistently negative throughout the course of the infection. The electrophoretic results are recorded in Table II.

TABLE II.—*Electrophoretic Analysis of Hamster Serum.*

	Day of infection.	Percentage. Albumen.	α-globulin.	β-globulin.	γ-globulin.	Remarks.
Infected animals untreated	10	51·8	21·2	15·9	11·3	..
	21	50·4	22·2	16·4	11·0	..
	28	49·4	27·4	16·2	7·0	..
	38	53·4	25·6	13·4	7·6	..
	50	32·7	44·7	13·5	9·1	Extracted 3 times with ether.
	63	28·8	49·3	13·1	8·8	Ditto.
	65	..	..	..	..	Three extractions with ether made serum protein insoluble.
	90	26·7	45·6	14·4	13·3	Extracted once with ether.
Infected animals treated with antimony	77	44·7	33·0	15·3	7·0	Extracted 3 times with ether.
	90	27·8	47·6	14·4	10·2	Extracted once with ether.
	105	25·5	49·3	16·1	9·1	Ditto.
Uninfected controls	0	53·8	22·7	14·3	9·2	..
	105	52·9	22·0	17·7	8·0	..

The above results were calculated from the descending pattern. Treatment with antimony was given on the 65th day of infection.

During the first six weeks the serum pattern of infected and control animals was similar. At the seventh week there was a marked decrease in the amount of albumin with an accompanying increase of α-globulin. The total protein concentration remained at the normal level. The lipaemia which occurred at this time possibly caused an increase in the β-globulin fraction in spite of the fact that the extracted sera showed normal values for the β- and γ-globulin throughout the infection. It should be noted that renal changes, including amyloidosis with accompanying oedema and proteinuria as described by Gellhorn *et al.* (1946), occurred at this time. It is recognized that such pathological lesions may give rise to a characteristic serum pattern (Luetscher, 1947). During the ninth week of infection the serum changes were maximal and persisted till the end of the experiment. The protein pattern of sera from the animals treated with sodium stibogluconate on the 65th day of infection and killed 12 days later had become practically normal whereas in animals similarly treated and killed 25 and 40 days later the serum showed the abnormal characters of the untreated animals. The return of serum abnormality was associated with a marked increase in the number of parasites in the spleen. Inoculations of spleen suspension from a normal animal and likewise administration of the antimonial drug to normal hamsters produced no change in their serum. The lipid content of the sera (Table III) was fairly constant till the seventh week after infection, when there was a marked increase in phospholipids, cholesterol and fatty acid, maximal at the 9th week, corresponding to changes in the serum pattern. After this the lipid content fell in untreated animals till the end of the experiment. In treated animals the values approached those for control animals so long as the infection was held in check, but deviated from normal as the number of parasites again increased. In the serum from two human patients with kala-azar there was an increase in the γ-globulin component, as found by other writers.

TABLE III.—*Serum Lipid Analysis of Hamsters.*

	Day of infection.	Mg. per c.c. Phospholipid.	Cholesterol.	Fatty acid.
Infected animals untreated	10	1·75	0·14	4·9
	21	—	—	—
	28	1·53	0·125	3·75
	38	2·05	0·094	3·1
	50	2·75	0·375	14·5
	63	3·55	0·47	16·5
	65	—	—	—
	77	2·75	—	—
	90	2·10	0·36	10·3
Infected animals treated with antimony*	77	1·55	0·15	7·4
	90	2·65	0·53	10·7
Uninfected controls	50	1·45	0·112	3·3
	90	1·95	0·15	5·0

* Treated with antimony on 65th day of infection.

DISCUSSION AND SUMMARY.

It is clear from these investigations that, during the course of visceral leishmaniasis in the golden hamster, the serum protein pattern undergoes a change which becomes marked when the infection has become severe. The question arises how far the changes recorded result from some activity of the parasites or are due to the associated renal conditions which invariably accompany heavy infections with *L. donovani* in the hamster. We have attempted to answer this question by treatment of the host with an antimonial drug active against the parasites. As a result of such treatment parasites could not be detected in spleen smears 12 days later ; and at the same time the serum pattern had returned to normal. Although effective against the parasites the drug was highly unlikely to have influenced the accompanying amyloidosis. It would appear, therefore, that the abnormal serum protein pattern, accompanied by an increase in phospholipids, cholesterol and neutral fat, was due to the activity of Leishman-Donovan bodies. This view is supported by the reappearance of the abnormal protein pattern when the few surviving parasites had multiplied and were again detectable in spleen smears. Whatever be the abnormal serum protein component (or components) responsible for the formol gel test in the human disease it does not appear to be present in hamsters, for the test was consistently negative in these animals. The protein pattern of hamsters with visceral leishmaniasis is abnormal in some respects, but it does not appear to be characteristic.

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REACTIVATION OF NEUTRAL MIXTURES OF INFLUENZA VIRUS AND SERUM BY VIRUS INACTIVATED BY HEAT.

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CALMETTE (1895) showed that a mixture of snake venom and antitoxin which was innocuous to animals on injection could be rendered toxic by heating at 68° C. for 10 minutes, a procedure which destroyed the antitoxin without affecting the toxin. This result was interpreted as evidence that the neutralization of toxin by antitoxin was not an irreversible process, a belief which has since been confirmed by many other workers. It may account for the results of Schmidt and Scholz (1929), who showed that diphtheria toxin-antitoxin floccules could be rendered toxic by addition of formol-toxoid, whereas the floccules and toxoid separately were innocuous.

The resemblance between the behaviour of toxin-antitoxin with that of virus and its antiserum has frequently been noted, and there is much evidence that the union of virus and antiserum is also, in many cases, a reversible one. Apparently neutral mixtures of a number of viruses and their antisera have been shown to be " reactivated " by simple procedures such as absorption on kaolin, dilution or ultracentrifugation, e.g. Todd (1928), Andrewes (1928), Sabin (1935). Recently McKee and Hale (1946, 1948) have shown that over-neutralized mixtures of influenza virus and antiserum, in which a considerable excess of antiserum was present, could be reactivated by addition of heat-killed virus. McKee and Hale suggested that reactivation could be used for the detection of influenza carriers in whom they supposed virus might be masked by the presence of antibody. In the present paper, results of an investigation into the mechanism of reactivation are described.

METHODS.

Viruses.—The strains were the standard PR8 strain of influenza virus A and the 1947 Kunz strain of influenza virus A (Stuart-Harris and Miller, 1947). Dilutions were made in 50 per cent broth-saline, a separate pipette being used for each dilution. Allantoic fluid was stored in rubber-stoppered tubes at — 76° C., and a separate tube was thawed for each experiment and then discarded.

Sera.—Antisera were prepared against each strain by inoculating a ferret intranasally with infected allantoic fluid and bleeding after 12–14 days. The

sera were heated at 56° C. for 30 minutes and stored at 4° C. The PR8 serum had an antihaemagglutinin titre of 1:10,000 to 1:20,000 against 4 minimal agglutinating doses of PR8 virus measured with a final concentration of 0·125 per cent fowl cells; the Kunz serum had a titre of 1:1500 to 1:2500 against Kunz virus.

Virus-serum mixtures.—Equal volumes of diluted virus and serum were mixed and allowed to stand at room temperature for one hour. Since the treatment thereafter varied in different experiments, the dilutions of serum and virus described in the text are the initial dilutions before mixing. The neutrality of a mixture was, in general, assumed when no haemagglutinin was demonstrated in the allantoic fluids from eggs inoculated at 11 days of incubation with 0·1 c.c. allantoically. In many cases the neutrality of the serum-virus mixture was also shown by lack of growth of virus on subinoculation into further eggs. This corresponds to an "absolutely neutral" mixture as described by McKee and Hale (1946). It was found that PR8 allantoic fluid diluted 10^{-3} when mixed with PR8 serum 1/200 for one hour at room temperature invariably produced a neutral mixture, whereas higher dilutions of serum often permitted growth of virus. Similarly Kunz allantoic fluid 10^{-5} was neutralized by Kunz serum 1/50 but not by serum 1/100. A slight excess of serum over the minimal neutralizing dose was provided in the neutral serum-virus mixtures used in the present experiments. An additional control of neutrality was provided in experiments such as that shown in Table II, in which reactivation of a mixture was effected by homologous but not by heterologous inactive virus.

Inactive virus concentrate (*I.C.*).—Concentrates of virus were prepared from infected allantoic fluid by absorption on 2 per cent fowl red cells at 4° C., and elution into approximately one-tenth volume of normal saline at 37° C. In the first experiment the concentrate was inactivated by heating at 56° C. for 30 minutes; allantoic inoculation into groups of eggs and two further passages from these showed no growth of virus. However, in later experiments and particularly with strain Kunz, certain concentrates were found which still contained live virus after 30 minutes at 56° C. In all experiments after the first, therefore, inactivation was carried out by heating at 56° C. for 90 minutes. As an additional precaution a test inoculation of concentrate diluted 1/10 was carried out, as two strains of virus have been encountered in which an auto-interference phenomenon occurred, i.e. virus heated at 56° C. for 60 minutes, gave no growth on allantoic inoculation, whereas the same material diluted 1/10 showed growth of virus. In most cases the agglutinin titre was not markedly diminished by heating at 56° C. for 90 minutes.

Inoculation of eggs.—Throughout these experiments allantoic inoculation of 11-day-old chick embryos was used. After inoculation the eggs were incubated at 37° C. for 48 hours, and were then chilled and the allantoic fluids harvested.

Haemagglutination titrations.—Harvested allantoic fluids were titrated by the Salk (1944) modification of the Hirst test with a final concentration of 0·125 per cent fowl red cells.

RESULTS.

In early experiments the technique of McKee and Hale (1946) was followed. One volume each of PR8 virus and antiserum were mixed and allowed to stand at room temperature for one hour. Two volumes of heat-inactivated virus were

then added, mixed, and allowed to stand two hours at room temperature. 0·2 c.c. volumes of the final mixtures were inoculated allantoically into groups of 11-day embryos. The inactive concentrate had a haemagglutinin titre of 15,000 and was used diluted 1:10 ; the concentration of serum was varied. The results are shown in Table I. The haemagglutinin titres in fluids where reactivation had occurred were those normally obtained in routine allantoic passage (1/2500–1/5000).

TABLE I.—*Reactivation of Neutral Mixtures of PR8 Virus with Varying Concentrations of Serum by PR8 Inactive Concentrate (I.C.).*

Dilution of virus.	PR8 antiserum dilution.	Reactivations* by I.C. of PR8 1/10.
10^{-3}	1/10	0/14 eggs positive
	1/50	10/15 ,, ,,
	1/100	3/10 ,, ,,

* In this and the following tables the right-hand column shows a fraction in which the numerator gives the number of eggs in which growth of virus occurred, and the denominator shows the number of eggs inoculated.

In some experiments the minimum amount of inactive concentrate was determined which was necessary to produce reactivation in the presence of constant concentrations of virus and serum. It was found that a mixture of PR8 virus 10^{-3} and serum 1/50 was reactivated by PR8 inactive concentrate (haemagglutinin titre 2560) diluted 1/10 but not diluted 1/50. Similarly, a neutral mixture of Kunz virus 10^{-6}, and serum 1/25 was reactivated by Kunz concentrate (haemagglutinin titre 1280) diluted 1/10 but not diluted 1/100.

Attempted reactivation in vitro *by heterologous virus.*

Since PR8 virus 10^{-3} plus antiserum 1/50 could readily be reactivated by addition of homologous inactivated virus, and since there was not a great excess of antiserum present, these concentrations were used to test the effect of addition of heterologous inactivated virus. Strain Kunz was used ; it is also a type A strain which was isolated in Sheffield in 1947, but is not closely related serologically to PR8 as shown by haemagglutination inhibition tests. Addition of a concentrate of inactivated Kunz virus did not cause reactivation of a mixture of PR8 virus 10^{-3} and serum 1/50 in any of 16 eggs. It did, however, cause reactivation of a neutral mixture of Kunz virus and serum (Table II).

TABLE II.—*Attempted Reactivation by Homologous and Heterologous Inactive Concentrate (I.C.).*

Virus and dilution.	Serum.	Reactivation by—	
		PR8 I.C.	Kunz I.C.
PR8 10^{-3}	PR8 1/50	5/5	0/6
Kunz 10^{-5}	Kunz 1/25	0/5	3/4

The following results show that no reactivation occurred with addition of heterologous inactivated virus to a neutral mixture of serum and virus even where there was only a very slight excess of serum present. It was found that

Kunz virus 10^{-5} was just barely neutralized by Kunz serum 1/50. Addition to the mixture of an equal volume of PR8 inactive concentrate gave growth of Kunz virus in 1 of 5 eggs ; growth also occurred in 1 of 5 control eggs in which an equal volume of broth-saline was added, an effect which can be ascribed to the dilution (Taylor, 1941). This fact has been repeatedly confirmed in later experiments ; thus the specific nature of the reactivating process with these two strains seems established.

Reactivation experiments in which the time of contact of the constituents was varied.

Burnet, Keogh and Lush (1937) showed that when mixtures of influenza virus and antibody kept in contact for various periods before test were inoculated on to the chorio-allantoic membrane, the proportion of survivors when the mixtures were three hours old was significantly reduced compared with that found with mixtures inoculated freshly. They suggested that this was due to the formation of a stronger union between virus and antibody. In the present experiments it was found that virus and serum kept in contact for 2 hours at room temperature was as easily reactivated as a mixture kept in contact for one hour (Table III(*a*)). In a further experiment a neutral mixture of PR8 virus and serum was kept at 4° C., and samples were removed after 1, 24 and 48 hours and reactivated *in ovo* by an equal volume of PR8 inactive concentrate. Table III (*b*) shows the results of these experiments. It can be seen that reactivation occurred up to 48 hours, the limit of time tested in this experiment.

TABLE III.—*Reactivation of PR8 serum-virus mixture after Varying Times of Contact of Antiserum and Virus.*

	PR8 virus dilution.	Antiserum dilution.	Time of contact of virus and antiserum.	PR8 I.C. added for—	Reactivations.
(*a*)	10^{-3}	1/50	1 hour	2 hours	3/5
	10^{-3}	1/50	2 hours	1 hour	4/5
(*b*)	10^{-3}	1/50	1 hour	..	2/5
	..	..	24 hours	..	1/4
	..	..	48 hours	..	5/5

Reactivation in ovo.

In an attempt to investigate further the mechanism of reactivation, experiments were carried out with inactive concentrates injected into eggs in advance of neutral mixtures of serum and virus, i.e. reactivation *in ovo* in contrast with reactivation *in vitro* carried out previously. The serum-virus mixtures were prepared as before and left in contact for one hour at room temperature, when 0·1 c.c. amounts were injected. Five to forty-five minutes before the latter inoculation (the actual interval of time varied in different experiments) 0·1 c.c. amounts of inactive concentrate were injected, both injections being made allantoically. Table IV shows these results.

It is seen that reactivation occurred when the inactive concentrate was injected first into the egg, and here again the reactivation was strain-specific. The fact that reactivation occurred when the constituents were brought together

TABLE IV.—*Reactivation* in ovo *with Inactive Concentrate Injected Before the Serum-Virus Mixtures.*

Virus.	Antiserum dilution.	Reactivation with—	
		PR8 I.C.	Kunz I.C.
Kunz 10^{-5}	1/25	0/5	3/5
PR8 10^{-3}	1/50	3/4	0/4

in the living allantoic cavity is of particular interest, since Anderson (1947) has shown that influenza virus which has been heated to destroy enzymatic activity is absorbed by red cells but cannot be eluted like unheated virus. Similarly it would be expected that inactive virus would be absorbed on to allantoic cells and would not be eluted. This was confirmed in the following experiments.

Absorption and elution of inactive virus in vitro *and* in ovo.

Concentrates of PR8 and Kunz viruses heated at 56° C. for 90 minutes were mixed with 2 per cent fowl's red cells and then left at 4° C. for 18 hours to allow absorption of virus to occur. The supernatant fluids were pipetted off and titrated for haemagglutinin and an equal volume of saline was added to the cells, which were then incubated at 37° C. for one hour without shaking. Thereafter, incubation at 37° C. was continued for 3½ hours, the cells being shaken at approximately half-hour intervals. The fluids were then centrifuged and the "eluates" pipetted off and titrated for haemagglutinins. The results of an illustrative experiment are shown in Table V (*a*), along with results obtained with unheated virus as a control. It is seen that the red cells absorbed the agglutinins from the heated concentrate, but that, unlike live virus, elution of inactive virus did not occur.

The fact that a similar mechanism occurs in the allantoic cavity was shown by an experiment in which 0·5 c.c. inactive virus was inoculated into the allantoic cavity of an 11-day embryo, and 0·2 c.c. samples of allantoic fluid were withdrawn after 5 minutes, 1 hour, and 2 hours, and titrated for haemagglutinins. As controls, live virus was inoculated and titrated similarly. The eggs were kept at 37° C. throughout. The results are shown in Table V (*b*).

TABLE V.—*Haemagglutinin Titres of Active and Heat-Inactivated Virus showing Absorption and Elution from* (a) *Fowl Red Cells,* (b) *Chick Allantoic Cells.*

Strain.	State of virus.	Haem-agglutinin titre.	After absorption on fowl red cells.	
			Supernatant titre.	Eluate titre.
(*a*) PR8	Active (control)	2560	320	1280
	Heated	320	< 20	< 20
Kunz	Active (control)	1280	160	1280
	Heated	1280	< 20	< 20

			Titre of allantoic fluid samples after		
			5 mins.	1 hour.	2 hours.
(*b*) PR8	Active (control)	2560	160	160	160
	Heated	2560	< 10	< 10	< 10
Kunz	Active (control)	320	40	10	< 10
	Heated	1280	< 10	< 10	< 10

Accordingly inactive virus injected into the allantoic cavity cannot be detected free after five minutes, whereas a significant proportion of live virus can be found in the allantoic fluid under similar conditions. Presumably the inactive virus is rapidly absorbed on to the allantoic cells and is not eluted. This raises the question of the mechanism of reactivation of a neutral serum-virus mixture by a preliminary injection of inactive concentrate into the egg. Since the inactive concentrate is rapidly absorbed on to the allantoic cells, the possibility must be considered that reactivation occurs at the surface of these cells. However, the effect of addition of antiserum to inactive virus which was already absorbed on to red cells was first examined. The technique used was as follows: 0·1 c.c. volumes of washed fowl cells were placed in a series of small centrifuge tubes and 1 c.c. volumes of Kunz I.C. (haemagglutinin titre = 1280) diluted 1/10 were added. Control cells received 1 c.c. saline. After incubation at 37° C. for 15 minutes the tubes were centrifuged and the supernatants pipetted off. The red cells were then thrice washed in saline and 1 c.c. volumes of different dilutions of Kunz antiserum were added. After one hour's incubation at 37° C. the tubes were centrifuged, and the supernatant fluids pipetted off and their antihaemagglutinin levels titrated against four minimal agglutinating doses of live Kunz virus. The deposited cells were then thrice washed, made up in saline to a 0·125 per cent concentration, and 1 c.c. volumes were allowed to settle at room temperature to see whether the cells were agglutinated. The results are shown in Table VI. As a control, one set of red cells to which inactive virus had been added received saline in place of Kunz antiserum; these control cells agglutinated on settling.

TABLE VI.—*Effect of Addition of Kunz Serum to Kunz Inactive Virus Absorbed on Fowl Cells.*

Red cells treated with—	Antiserum dilution.	Antihaemagglutinin titre of supernatant (in terms of undiluted serum).	Agglutinability of red cells on settling.
Inactive virus	1/25	1:1600	Not agglutinated
Saline control	1/25	1:1600	,, ,,
Inactive virus	1/50	Just < 1:800	,, ,,
Saline control	1/50	1:800	,, ,,
Inactive virus	1/250	< 1:500	,, ,,
Saline control	1/250	1:1000	,, ,,
Inactive virus	Saline	—	Agglutinated

Thus, on adding antiserum to inactive virus absorbed on red cells, the concentration of antihaemagglutinin in the supernatant fluid showed definite reduction when the higher dilutions of antiserum were used; also the red cells no longer agglutinated on settling. These results could be explained by assuming that inactive virus was removed from the red cells by the antiserum, thus reducing its antihaemagglutinin titre, or alternatively that the inactive virus remained

on the red cells absorbing serum antibodies from the supernatant. Experiments were also carried out to determine whether the same result would occur when the inactive virus and serum were mixed first of all and then the mixture added to the red cells. It was found both with PR8 and Kunz viruses that the order of addition of inactive virus and serum made no difference to the final result.

Reactivation in vitro *using inactive virus absorbed on red cells.*

The reactivation technique was made use of in order to determine whether inactive virus remains fixed to red cells on addition of antiserum. Two c.c. of PR8 inactive concentrate (titre 2560) were absorbed on to 0·2 c.c. fowl red cells. The cells were then thrice washed in saline and 2 c.c. of a neutral mixture of PR8 10^{-3} and serum 1/50 were added. The mixture was incubated at 37° C. for one hour and then centrifuged. The supernatant was pipetted off and the deposited cells made up to 2 c.c. in broth saline. The supernatant and deposit in 0·2 c.c. amounts were inoculated allantoically into 11-day embryos. The results in Table VII (*a*) show that inoculation of eggs with both the supernatant and the deposit led to growth of virus.

TABLE VII.—*Reactivation of a Neutral Mixture of Serum and Virus by Addition in vitro of Inactive Virus Absorbed on Red Cells.*

I.C.	PR8 10^{-3} + antiserum 1/50	Results with inoculation of—	
		Supernatant.	Deposit.
(*a*) PR8	2 c.c.	6/6 positive	6/6 positive
(*b*) PR8	1 c.c.	5/5 ,,	5/5 ,,
Kunz	1 c.c.	0/5 ,,	0/5 ,,

In order to show that such reactivation was due specifically to the inactive virus concentrate and not, e.g., to any property of the red cells, inactive concentrates of PR8 and Kunz respectively (adjusted to the same agglutinin titre) were absorbed on to fowl red cells. Reactivation of a neutral mixture of PR8 virus and serum was then attempted with these two concentrates in the same manner as above. Reactivation was obtained only with the homologous virus concentrate (Table VII (*b*)).

These results show that reactivation has occurred both on the deposited red cells and in the supernatant fluid, and that the reactivation did not occur when inactive PR8 virus was replaced by heterologous virus. It is difficult to escape the conclusion that some inactive virus was removed from the red cells by the antiserum. Since this work was carried out, Dr. W. J. Elford and Dr. I. M. Dawson, of the National Institute of Medical Research, Hampstead, have confirmed by means of electron microscope studies that inactive PR8 virus absorbed on to fowl red cells is in part removed on addition of homologous antiserum (unpublished personal communication). No evidence was obtained from these and similar experiments with Kunz virus that the virus-serum mixtures were more easily reactivated by inactive virus in the immediate neighbourhood of the red cells (that is in the deposit) than in the supernatant fluid.

Reactivation in ovo *by preliminary injection of a small amount of inactive concentrate.*

It was of interest to determine whether reactivation would be more effective if inactive concentrate was injected before rather than along with a serum-virus mixture. In order to test this point, diluted inactive concentrate was used. Reactivation of a neutral mixture of Kunz 10^{-6} and serum 1/25 was carried out with a preparation of Kunz I.C. diluted 1/10. Two groups of eggs were inoculated. In the first group the I.C. was injected 5 minutes before the virus-serum mixture, and in the second group it was injected along with the virus-serum mixture; otherwise both groups were alike. In the first group 5 out of 17 eggs and in the second group 1 out of 15 eggs showed reactivation. Similar experiments carried out with PR8 also showed slightly better results when the inactive concentrate was injected first. PR8 virus 10^{-3} + serum 1/50 was reactivated with PR8 concentrate (titre 2560) diluted 1/10. Altogether 8 out of 8 eggs of the first group and 4 out of 8 eggs of the second showed reactivation.

The slight advantage found when the inactive virus was injected before the virus and serum was also shown in the following experiment. Two groups of eggs were used. In one group an injection was given of inactive virus, followed after 15 minutes by an injection of 0·1 c.c. fowl red cells. In a control group of eggs a preliminary injection of saline was given, followed after 15 minutes by the same amount of inactive virus absorbed on 0·1 c.c. of red cells. After a further 15 minutes both groups of eggs received 0·1 c.c. of a neutral serum-virus mixture. Again, the same quantities were used in each group, only the order of injection being varied. The results are shown in Table VIII.

TABLE VIII.—*Comparison of Reactivation by Inactive Virus Absorbed on Red Cells and Inactive Virus Injected Before the Red Cells.*

Virus.	Antiserum.	I.C.	Reactivation occurring after	
			I.C. injected first.	I.C. injected absorbed on R.B.C.
PR8 10^{-3}	1/50	PR8	5/5	3/5
Kunz 10^{-5}	1/25	Kunz	5/9	2/10

Thus in all four experiments it was found that reactivation was more effective when the inactive virus was injected before rather than along with a serum-virus mixture. Individually these experiments give values for P of 0·116, 0·038, 0·130, 0·222. Taken together they give a value of P = 0·00013 (the product of the individual values). It thus appears to be very unlikely that the results are due to chance.

DISCUSSION.

The neutrality of a mixture of virus and antiserum is not easy to define. According to the "percentage law" (Andrewes and Elford, 1933) the product of the concentration of serum and the percentage of survivors is constant. This law, first recognized in regard to the behaviour of a bacteriophage with its antiserum, has been found to apply to the reaction between a number of viruses and their antisera (Burnet, Keogh and Lush, 1937). Thus a "neutral" serum

virus mixture is one in which the number of survivors is insufficient to produce a recognizable infection, according to the method used to demonstrate the latter. For example a serum-virus mixture can be prepared which will not show multiplication of virus when injected intranasally into mice, but will still produce infection in the more sensitive chick embryo. Since the mixtures used in the present experiments were tested by allantoic inoculation of chick embryos, a neutral serum-virus mixture is defined as one which did not lead to the development of haemagglutinins when 0·1 c.c. was inoculated allantoically into 11-day embryos. Such neutral mixtures could readily be reactivated by concentrated homologous inactivated virus as reported by McKee and Hale (1946). The reactivation exhibited serological specificity, however. The strains PR8 and Kunz were chosen as type A influenza strains which showed considerable serological divergence in cross-haemagglutination-inhibition tests but some common antigenic structure also (Stuart-Harris and Miller, 1947). No evidence of cross-reactivation was found even when a barely neutral mixture of serum and virus was used.

It was also found that reactivation occurred quantitatively. Thus a neutral mixture of PR8 virus and serum which was reactivated by a 1/10 dilution of PR8 inactive concentrate, was not reactivated if the concentration of antiserum was increased five times or the inactive concentrate used at 1/50 dilution. Similar results were found to hold for Kunz virus also. Accordingly the inactive virus would appear to combine specifically and quantitatively with the serum antibodies and displace them from the live virus.

Henle and Henle (1944) have shown that influenza virus inactivated by ultraviolet light or heat can interfere with the propagation of live virus in the developing chick embryo. In the concentrations used in the present experiments, however, inactive virus (heated at 56° C. for 90 minutes) when added to live virus in the absence of serum did not interfere with multiplication. Homologous and heterologous inactive concentrates behaved alike in this respect. On varying the order of addition of reagents, it was found that inactive concentrate injected into eggs before a neutral serum-virus mixture still caused reactivation. Experiment has shown that inactive concentrate injected into the allantoic sac is rapidly taken up by the allantoic cells and is not subsequently eluted, its behaviour being analogous to what is observed after absorption on to fowl red cells. It thus seemed likely that inactive virus absorbed on to the surface of the allantoic cells could react with serum antibody molecules coating live virus particles. In this way a redistribution of the antibody molecules would occur as a result of which particles of live virus released from antiserum would be free to infect the allantoic cells.

Two groups of experiments have been devised to test the possibility that reactivation occurred on the surface of the allantoic cells. In the first the approach was indirect. It was shown that *in vitro* a neutral serum-virus mixture could be reactivated by homologous inactive concentrate absorbed on red cells; also specific reactivation was demonstrable not only in the immediate neighbourhood of the red cells, but also in the supernatant fluid obtained after centrifugation. The conclusion was reached that antiserum removed some inactive virus from the red cells, and by analogy, the same result would occur in the allantoic sac.

A more direct approach consisted in comparing the success of reactivation by diluted inactive concentrate when injected into the allantoic sac before and

along with a neutral serum-virus mixture. The results showed slightly more successful reactivation when the inactive concentrate was injected before the serum-virus mixture. The difference was found in four experiments, and statistically the results are highly significant ($P = \cdot 00013$).

A possible explanation of these results rests on the assumption that reactivation occurs according to the "law of mass action." When inactive virus is injected first it is absorbed on to the allantoic cells, but is in part removed on subsequent addition of antiserum. Presumably this "eluting" action of serum is quantitative and occurs until an equilibrium is set up between serum, virus and allantoic cells. When a relative excess of inactive concentrate is present on the allantoic cells a mixture capable of releasing live virus would be formed both in the allantoic fluid and in the neighbourhood of the allantoic cells. It can easily be supposed that live virus actually on the surface of the allantoic cells would then have a better chance of initiating infection. Whether the allantoic cells participate actively in the process is not yet settled.

SUMMARY.

(1) Neutral mixtures of two strains of influenza virus A with their respective antisera could be reactivated *in vitro* by addition of homologous but not heterologous heat-inactivated virus.

(2) Inactive virus absorbed on fowl red cells did not elute spontaneously at 37° C., but could be partially "eluted" by addition of homologous antiserum.

(3) Reactivation could be produced *in ovo*, i.e. by injecting homologous inactive virus 5–45 minutes before a neutral serum-virus mixture.

(4) Reactivation *in ovo* was slightly more effective than reactivation *in vitro*.

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CHANGES IN THE METAPHYSIS OF THE LONG BONES DURING THE DEVELOPMENT OF RICKETS.

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THE changes in the epiphyseal cartilage and the formation of the metaphysis in rickets have been very fully described by Park (1939) and Dodds and Cameron (1934, 1938, 1939). In essence, calcification of the epiphyseal cartilage stops, as does normal cartilage removal, and the hypertrophic layer of cartilage cells increases greatly in width. Later, division of the parent cartilage cells ceases, and the existing cartilage trabeculae are overlaid with osteoid which comes into direct contact with the hypertrophic cartilage cells. Subsequently, in certain areas, a process which Dodds and Cameron (1934) call " renewed cartilage removal " takes place. Here large tongues of marrow cut into the uncalcified cartilage in all directions over a broad front, and cartilage is removed in an indiscriminate manner without preliminary calcification, and replaced by osteoid.

It seemed that further information on this subject might be obtained by causing a slight degree of healing and then returning the animal to the rachitic state, the calcified zone acting as a marker in the metaphysis during the subsequent observations. Such a calcified line of healing can be induced by administration of vitamin D, adjustment of the Ca : P ratio of the diet and by the addition of P (Karelitz and Shohl, 1927 ; Key and Morgan, 1932 ; Bruce and Callow, 1934), or starvation (McCollum, Simmonds, Shipley and Park, 1922*a* ; Kramer, Shear and Siegel, 1931). The healing produced by vitamin D or starvation is usually present in the early stages as a line of calcified cartilage at the cartilage-metaphysis junction (Dodds and Cameron, 1938). This calcified line forms the basis of the well-known line test, originally proposed by McCollum, Simmonds, Shipley and Park (1922*b*). Addition of P to the diet also causes a calcified line in the same area as well as irregular calcification in the metaphysis (Irving, 1946*a*).

Owing to the fact that addition of P to the diet or dietary restriction causes healing to occur quickly, whereas the action of vitamin D takes at least 3 days to manifest itself and continues for some time after administration (Irving, 1944), the two former methods were considered to be more suited to the type of experiment planned. Animals with well-marked rickets were therefore either starved for a short period and then returned to the rachitogenic diet, or else were given a diet of normal Ca and P content for a short time and were then given again the rachitogenic diet. These procedures caused the appearance of a narrow calcified area in the epiphysis, which persisted and subsequently moved down the metaphysis as this slowly grew.

EXPERIMENTAL.

Two series of young albino rats of the Wistar Institute strain were used, rickets being developed by a preliminary period of 28 days on the Steenbock and Black (1925) diet no. 2695 (Diet A, Table I).

TABLE I.—*Composition of the Diets Used.*

Diet.	Ca%.	P%.	Ca : P.
A	1·39	0·25	5·6
B	1·43	0·77	1·86

Group I consisted of 40 animals (6 litters). After the 28 days' preliminary period one animal of each litter was killed and examined. The dietary intake of the other rats was then restricted so that they each lost on an average about 4 g. per day. After 6 days of dietary restriction one rat of each litter was killed and examined and the rest were again fed to appetite. The subsequent times of killing are given in Table II.

TABLE II.

Treatment.	Serum calcium mg. per 100 c.c.
Group I :	
28 days on diet A	12·34
6 days' dietary restriction	9·21
Diet A fed to appetite for—	
3 days	10·88
6 ,,	11·50
9 ,,	12·29
12 ,,	11·64
15 ,,	12·42
18 ,, (one rat)	12·50
21 ,, (,,)	12·98
Group II :	
28 days on diet A	14·60
3–6 days on diet B	9·08
Diet A fed for—	
2 days	11·54
3 ,,	10·44
4 ,,	11·73
6 ,,	11·32
8 ,,	13·66
9 ,,	11·90
11 ,,	11·50
12 ,,	11·03
14 ,,	10·97

Group II consisted of 26 rats (4 litters). One rat of each litter was killed and examined after the preliminary period of 28 days. The rest of the litter were given for 3–6 days a modified Steenbock and Black diet to which sufficient P as Na_2HPO_4 had been added to raise the P content to 0·77 per cent (Diet B,

Table I). At the end of this period another rat of each litter was killed and examined. The rest of the litter was returned to diet A, and one rat of each litter was killed in accordance with the data given in Table II.

Weighing of the rats.—During the preliminary 28-day period on the rachitogenic diet A the rats were weighed once weekly. They were weighed daily during the rest of the experiment.

Examination of the rats.—The serum Ca values were determined at death by the iodometric method of de Loureiro and Janz (1944). The lower ends of one radius and ulna and the upper end of one tibia were examined by the "line test" method (McCollum, Simmonds, Shipley and Park, 1922*b*; Coward, 1947), and the same areas of the corresponding other bones were prepared for histological examination by the modification of von Kossa's method described by McLean and Bloom (1940). Various measurements in the epiphysis and metaphysis were undertaken by means of a micrometer eyepiece.

RESULTS.

Appearance and growth of the animals.

After the preliminary period on diet A all rats had well-developed rickets, as shown by palpably swollen epiphyses. The short healing period made no obvious difference to the animals. After return to diet A both groups of animals continued to look healthy for a while, but after 7–9 days some of the animals became unthrifty, and subsequently a few developed paralyses and urinary incontinence and had to be sacrificed. The development of paralyses after long continuation on a rachitogenic diet has been described by Boer, Arons and van der Rijst (1937) and by Shapiro (1941).

During the preliminary period on diet A, as is usually found, the growth of the animals was subnormal, varying from an increase of 0·5 to 1·5 g. per day.

Group I.—During the 6-day period of dietary restriction the rats lost from 11 to 28 g. in weight. One-half to one-third of this weight loss occurred on the first day, when no food was given. After diet A was re-fed in unrestricted quantities there was a marked gain in weight on the first day (usually about 10 g.). This resumption of growth did not continue, however, and for the remainder of the experimental period there was little gain in weight, only one animal regaining its pre-starvation weight after 12 days. None of the other rats surviving as long or longer than 12 days did so, although some of them approached it.

Group II.—This group of animals tended to lose weight while on diet B, but this loss of weight was not sufficient to account for the healing which occurred, nor was the healing of the type found after starvation. The growth occurring when diet A was later substituted was slight. After a few days the weights of these animals remained more or less constant, and seldom exceeded the weights before transfer to diet B.

Serum Ca values.

These were high at the end of the preliminary period on diet A, and had fallen somewhat at the end of the healing period. On return to diet A the values rose to their original high levels. Averages of these values are given in Table II.

Line tests.

All animals of both groups had florid rickets with wide epiphyses after one month on the rachitogenic diet.

At the end of the period of dietary restriction the Group I animals showed a typical line of healing, more marked in the tibiae than in the radii and ulnae. The Group II rats also showed calcification in the same area, and a certain amount of diffuse calcification throughout the metaphysis (Fig. 1). After cessation of starvation or return to diet A, the calcified cartilage at the epiphyseal cartilage-metaphysis junction behaved similarly in both groups. Calcification stopped abruptly, but the area already calcified remained as such. With the passage of time the calcified cartilage moved away from the epiphyseal cartilage into the metaphysis towards the shaft of the bone. This was visible after three days (Fig. 2), and, while movement into and through the metaphysis was variable, presumably depending on the individual response of each rat to treatment, the general tendency was for a slow, steady movement of the calcified zone (Fig. 3). This zone usually retained its linear appearance in the early stages. The tibia of one animal of Group I killed after 15 days showed a diffuse deposit of calcified material in the lower part of the metaphysis, as did those of two animals that survived to the 18th and 21st days respectively. In the radii and ulnae, however, the deposit was still linear at this stage, and in no case did it appear as near the diaphysis as it did in the tibiae.

EXPLANATION OF PLATES.

All figures are of the upper end of the tibia. With the von Kossa technique calcified tissues appear black.

FIG. 1.—Rat 1096, showing healing imposed on the rachitic state after 4 days' consumption of diet B. Note the line of healing along most of the epiphyseal cartilage-metaphysis junction and some irregular calcification in the metaphysis. (× 10.)

FIG. 2.—Rat 1098. Healing of rickets was caused by the consumption of diet B for 5 days. The animal was then returned to diet A and killed 3 days later. The epiphyseal cartilage and the calcified cartilage are separated by a zone of marrow. The sharpness of the upper edge of the calcified cartilage shows that calcification stopped very abruptly. (× 10.)

FIG. 3.—Rat 1100. This animal was treated similarly to no. 1098, but was killed 9 days after return to diet A. Note that the epiphyseal cartilage and the calcified cartilage are now more widely separated by a marrow zone. (× 10.)

FIG. 4.—Rat 1097. Calcification of the rachitic cartilage at the epiphyseal cartilage-metaphysis junction caused by 5 days' subsistence on diet B. (× 240.)

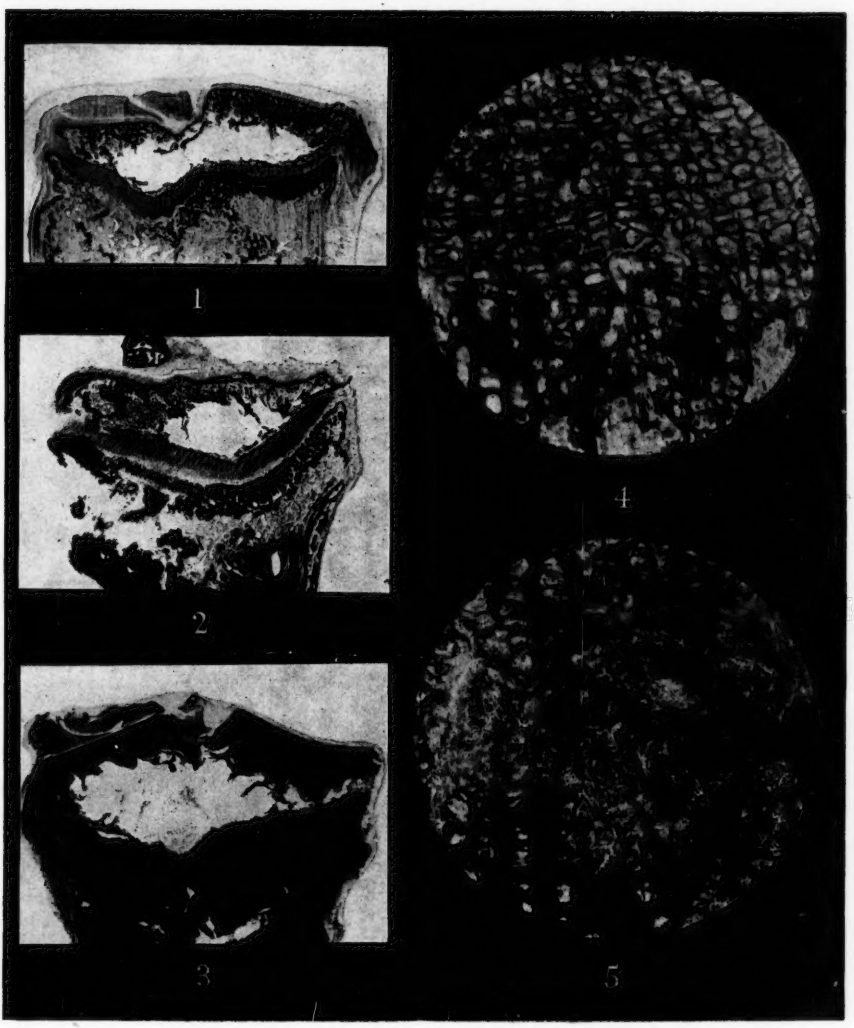
FIG. 5.—Rat 1104. This animal was treated similarly to no. 1098, but was killed 2 days after return to diet A. Capillaries and marrow cells have invaded through the calcified cartilage and pushed it back from the rest of the epiphyseal cartilage. Osteoid is being already laid down on the calcified cartilage. (× 240.)

FIG. 6.—Rat 1101. This animal was treated similarly to no. 1104, but was killed 12 days after return to diet A. The separation of calcified cartilage and epiphyseal cartilage is not here so great. The epiphyseal cartilage has been compressed in places by the osteoid formed round the calcified cartilage. (× 240.)

FIG. 7.—Rat 1106. Treated similarly to no. 1104, but killed 6 days after return to diet A. Here the separation of epiphyseal cartilage and calcified cartilage, is considerable and osteoid trabeculae have formed in this space. (× 90.)

FIG. 8.—Rat 1109, treated similarly to no. 1104, but killed 11 days after return to diet A. The calcified cartilage has been broken up to some extent by invading capillaries and marrow cells, and also by the splaying out of the cartilage trabeculae probably due to mechanical stresses. (× 90.)

FIG. 9.—Rat 1051. This animal had had its dietary intake restricted for 6 days after the development of rickets and was then re-fed diet A to appetite for 6 days. The calcified cartilage is partly in a tongue of cartilage and partly in the metaphysis. Renewed cartilage removal has taken place on either side of the field but not in the middle. The line of calcified cartilage is continuous from metaphysis to epiphyseal cartilage. The V-shape is due to the similar shape of the epiphysis. (× 90.)



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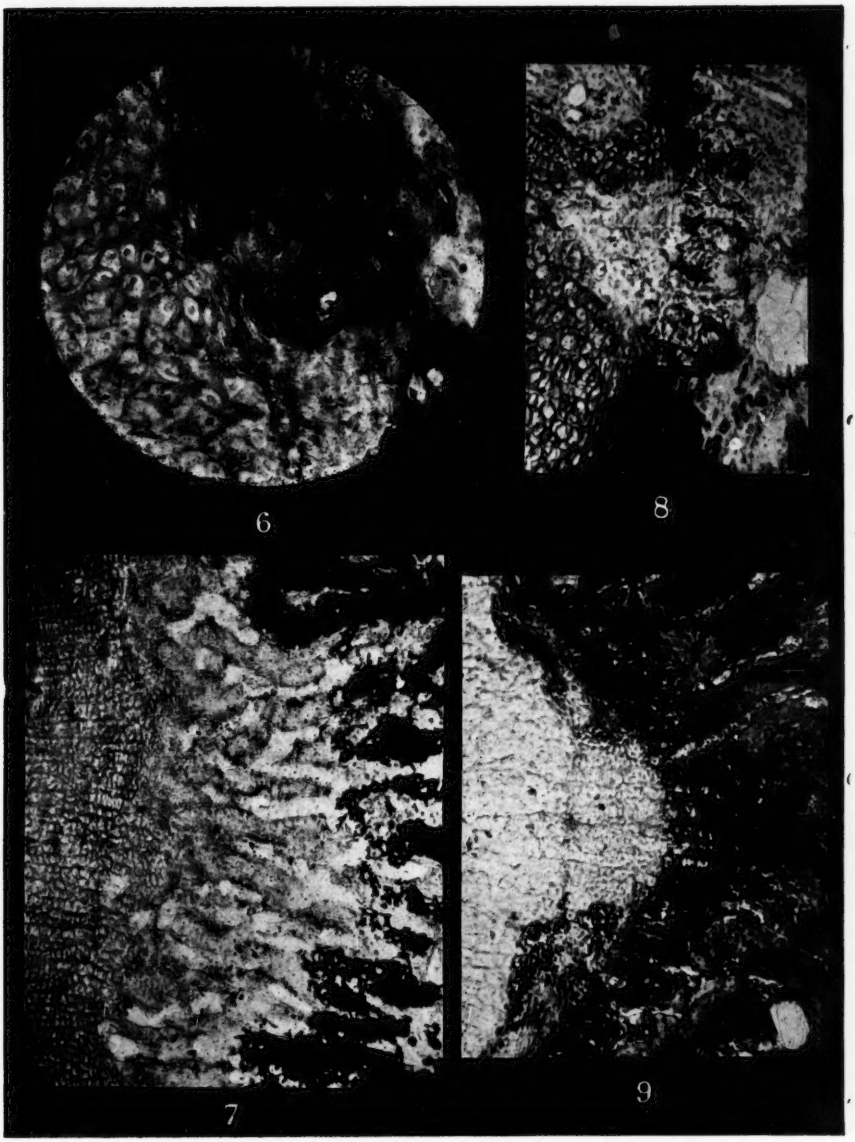

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Histological examination.

The histological findings confirmed the interpretation of the bones examined by the line test method.

Sections from bones of animals of both groups killed after the preliminary 28 days on diet A showed the characteristically wide, irregular epiphyseal cartilage of rickets, with long projections of hypertrophic cartilage cells reaching far into the metaphysis in places, and covered with osteoid. In the tibia "renewed cartilage removal" (Dodds and Cameron, 1934) was particularly conspicuous. In places the cartilage was of full thickness and abutted on to the shaft, while elsewhere broad tongues of marrow had invaded deeply into the cartilage and reduced its width considerably. The trabeculae of the spongiosa were surrounded by osteoid borders.

After the period of dietary restriction of the Group I rats, early stages of the healing process were present at the cartilage-metaphysis junction. Granular deposits occurred in the intercellular matrix of the hypertrophic chondrocytes; an invasion of the degenerating cartilage by connective tissue and capillaries had started; where long narrow ingrowths of cartilage into the metaphysis were present these tended to show strong calcification, and uncalcified hypertrophic cells with intact walls often surrounded areas of calcified chondrocytes. Similar changes were found in the Group II animals after the period on diet B (Fig. 4); in addition, a certain amount of scattered calcification developed throughout the metaphysis. The findings to be described concern only the behaviour of the calcified cartilage at the epiphyseal cartilage-metaphysis junction, and here the same changes occurred in both groups.

Directly starvation stopped, or the animals were returned to diet A, the calcification process ceased abruptly. This histological finding corresponded to the almost immediate return of the blood Ca to its original higher level. Within the next few days small tufts of marrow developed between the epiphyseal cartilage and the calcified cartilage, and "renewed cartilage removal" was resumed (Fig. 5); shortly after, osteoid formed in this area. The calcified cartilage soon became itself a focus for osteoid formation, which enveloped it more and more as the experiment proceeded. In some cases the epiphyseal cartilage was forcefully compressed by the amount of osteoid formed around the calcified cartilage (Fig. 6). Subsequently more marrow and osteoid formation took place between the calcified cartilage and the edge of the epiphyseal cartilage and the calcified cartilage was slowly pushed through the metaphysis (Fig. 7). The marrow appeared to penetrate into the area of cartilage removal via the openings in the calcified cartilage made by invading capillaries when the short period of calcification had taken place. Subsequently these openings appeared to enlarge, and later, especially in the tibia, the calcified line became somewhat broken up (Fig. 8). This was due to osteoid formation around the line, to mechanical invasion by the marrow and to physical stresses but not to absorption. The edges of the cells remained at all stages as outlined by the calcified intercellular matrix. The degree of calcification did not alter, and the intercellular matrix was not broken down or invaded. Thus the calcified cartilage very probably behaved much like uncalcified cartilage not subjected to "renewed cartilage removal," but was much better visualised in virtue of the fact that it was calcified. Dodds and Cameron (1934) have shown that osteoid is deposited on persisting trabeculae of cartilage in the metaphysis.

Measurements of the distance between the calcified edge of the epiphysis and the calcified cartilage at different stages showed that the latter moved slowly and somewhat irregularly through the metaphysis away from the epiphysis. The width of the epiphyseal cartilage during this period stayed roughly constant at about 280μ in all three bones, indicating either that the proliferation of cartilage had stopped, or that renewed cartilage removal was keeping pace with proliferation. Thus the movement of the calcified cartilage was not caused by changes in the width of the epiphyseal cartilage, but by the increase in marrow width on the epiphyseal side of the calcified cartilage.

In certain areas, especially in the tibia, the hypertrophic cartilage cell layer was very wide and in contact with the shaft. In such places " renewed cartilage removal " had not occurred on the side facing the shaft. Park (1939) attributes this to the protection exerted by a barrier of crushed cartilage cells and matrix framework. The calcified cartilage line in such areas was situated in the cartilage mass (Fig. 9), and slowly moved towards the shaft, keeping pace with the calcified continuation of the line which was embedded in the osteoid metaphysis. This showed that the cartilage of these masses was still growing. The probability thus exists that the cartilage referred to above, which was undergoing " resumed removal," was in fact also growing, and at the same rate as it was removed.

" Renewed cartilage removal " also occurred on the lateral faces of the large cartilage masses, undercutting them as had been described by Dodds and Cameron (1934). This removal did not affect the calcified cartilage, which was avoided and left unaltered. This confirms the findings of Dodds (1932) that marrow cells cannot remove calcified cartilage, for which process osteoclasts are needed.

DISCUSSION.

The calcification which occurred as a result of dietary restriction or addition of P to the rachitogenic diet was the same as that previously reported, and was found predominantly either at the cartilage-metaphysis junction, or at the same level across the broad bands of adjacent cartilage. Müller (1858) and several subsequent writers have stated that it occurred where calcification would have normally taken place, but, as with Dodds and Cameron (1938), the present writers found that the cartilage-metaphysis junction was the invariable situation for calcification. This accounts for the finding of calcification at various levels in the metaphysis, depending on the width of the epiphyseal cartilage and the amount of " renewed cartilage removal " going on.

On the return of the animals to the rachitogenic diet calcification ceased very abruptly. In spite of the period of healing, the rats were still in a sufficiently " rachitic condition " for florid rickets to be re-established at once. The teeth of these animals reacted in a similar way. Dentin calcification immediately ceased and enamel formation also stopped abruptly within a few hours of transfer back to the rachitogenic diet (Irving, 1946*b*).

This cessation of calcification was visible at first as a cessation of the penetration of capillaries into the hypertrophic chondrocytes, and shortly after, tongues of marrow insinuated themselves between the calcified cartilage and the edge of the epiphyseal cartilage. This is interpreted as a resumption of " renewed cartilage removal," and the writers consider that this process kept the epiphyseal cartilage at constant width in most areas of the epiphysis, and

that under the present experimental conditions cartilage proliferation never completely ceased. This is at variance with the conclusions of Dodds and Cameron (1934), who considered that cartilage growth ceased after about 3 weeks on the rachitogenic diet. It might be argued that the short break in the rachitic regime was sufficient for cartilage proliferation to begin afresh, but the very rapid resumption of the rachitic state subsequently makes this explanation unlikely.

Dodds and Cameron (1943) state that the growth of the rachitic bone after 3 weeks is solely due to widening of the epiphyseal cartilage. Since this statement is based on X-ray findings, it would appear that both the metaphysis and the epiphyseal cartilage are included in the term "epiphyseal cartilage." In the present experiments the epiphyseal cartilage, apart from the very wide masses, appeared to remain at constant width, and the growth of the bones must therefore have been solely due to increased osteoid formation. This almost certainly accounts for the progress of the calcified cartilage through the metaphysis. Two days after return to the rachitic condition marrow and osteoid formation had pushed the calcified cartilage back from the hypertrophic cartilage cell layer, and as the experiment proceeded this went on continuously. Thus the process of "renewed cartilage removal" must be accompanied by the formation, on the site of the removed cells, of osteoid masses. The site of growth of the bone had not been changed, but the process had been completely altered, osteoid being deposited continuously in place of the removed cells. It may well be that the cells are not all removed, but that some persist as nuclei for the deposition of osteoid. This is, however, difficult to see, owing to the thickness of the osteoid masses.

The persistence of the calcified cartilage cells can be partially explained by the protective layer of osteoid formed over them. This calcified material seemed to be particularly attractive to osteoid formation, and in some cases the hypertrophic cartilage cells were pushed on one side by the amount of osteoid formed. Since osteoclasts are unable to attack osteoid (Dodds, 1932; Weinmann and Schour, 1945), such as were present in the marrow could not get access to calcified tissue. The marrow tufts on the other hand have been shown by Dodds (1932) to be unable to remove calcified material. Thus, even when the calcified cartilage was subjected to the influence of the marrow, as was seen in the lateral invasion of the wide cartilage masses, it was left unchanged.

SUMMARY.

1. The changes in the metaphyses of rachitic rats have been investigated after causing a narrow strip of calcification for a short period by either starvation or adding phosphate to the rachitogenic diet.

2. After return to the rachitogenic diet calcification ceased abruptly, and the calcified strip of cartilage was soon separated from the epiphyseal cartilage by invading marrow. This calcified tissue was quickly covered by osteoid.

3. The invading marrow broke up the calcified cartilage mechanically to some extent, but the degree of calcification was not altered, nor was the intercellular matrix broken down or invaded.

4. As the experiment proceeded, the calcified cartilage slowly moved away from the epiphysis towards the diaphysis.

5. Under the conditions of the present experiments proliferation of the epiphyseal cartilage did not stop, but the process of "renewed cartilage removal" (Dodds and Cameron, 1934) kept the epiphyseal cartilage at constant width.

6. The growth of the bones during rickets occurs at the epiphyseal cartilage-metaphysis junction, where fresh osteoid is laid down as the process of "renewed cartilage removal" takes place.

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MENGO ENCEPHALOMYELITIS VIRUS: ISOLATION AND IMMUNOLOGICAL PROPERTIES.

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The purpose of this paper is to describe the isolation of a virus, believed to have been previously unknown, and to present the preliminary immunological studies which have been made with it. The virus has been named Mengo encephalomyelitis virus because it was isolated in the Mengo District in Buganda, Uganda, and because it can produce lesions in the brains and spinal cords of infected animals (Dick, 1948), and encephalitic symptoms in man (Dick, Best, Haddow and Smithburn, 1948).

Isolation of Virus.

Mengo encephalomyelitis virus (MEV) was first isolated from a paralysed rhesus monkey of the laboratory stock, which had not been subjected to any experimental procedures. It was subsequently isolated from a mixed batch of mosquitoes of the genus *Taeniorhynchus*, from a batch of *Taeniorhynchus* (*Coquillettidia*) *fuscopennatus* Theobald mosquitoes, from a mongoose (*Ichneumia* sp.), and from a human case. About a year after the first isolation a sixth strain of the virus was isolated from another of the laboratory stock of unused rhesus monkeys. Apart from some slight variations in the incubation period, all these viruses and the virus isolated from the human case of Mengo encephalomyelitis produced identical signs in inoculated mice (Dick, 1948). Evidence will be introduced to show that these 6 strains of virus are identical.

Isolation from a paralysed rhesus monkey.

On June 19, 1946, a rhesus monkey (UR 22) from the reserve stock of monkeys was brought into the laboratory because it did not look well. The stock of monkeys from which UR 22 came is kept in open-air runs in the compound of the institute. Rhesus UR 22 had a normal temperature. Three days later it was found to have a flaccid paralysis of both legs and slight weakness of the right arm. Specimens of its blood and cerebrospinal fluid were taken, and groups of mice were inoculated intracerebrally with these materials. A group of mice consisted of 6 animals which, under routine, were caged together on weaning at 21 days of age. The mice employed in all experiments were descended from the original stock of Swiss white mice from Carworth Farms near New York City. At the time of use in experiments mice were between 35 and 42 days old unless otherwise stated. The monkey was then sacrificed. Areas of slight congestion were found in the lower lumbar region of its spinal cord, but apart from that no gross pathological changes were observed in any of the viscera.

Portions of the cortex, midbrain, pons, medulla, cerebellum, and of the cervical, upper thoracic, lower thoracic and lumbosacral regions of the cord were taken for inoculation of mice and for histopathological examination. Each portion of brain and cord was ground separately in normal saline to make an approximate 10 per cent suspension. All suspensions and dilutions referred to in this paper are in terms of weight to volume, and do not take into account loss by centrifugation, filtration or other procedures. These suspensions were centrifuged at low speed for 10 minutes, and the supernates, both Seitz-filtered and unfiltered, were injected intracerebrally into groups of 6 adult mice and intraperitoneally into 4 mice 4 days old.

Mice inoculated with the blood serum of UR 22 and with suspensions of its cortex, pons, cerebellum and medulla remained well throughout an observation period of 30 days. Of the 6 mice inoculated with the cerebrospinal fluid of the monkey, 1 became paralysed on the 8th day and died on the 11th day after inoculation ; the other mice of this group remained well. Among the 6 inoculated with the Seitz-filtered suspension from the midbrain, 1 became paralysed on the 6th day and died on the 8th day. Of the 6 adult mice inoculated with the unfiltered supernate of the suspension of the cervical and upper thoracic portions of the spinal cord, 2 were paralysed on the 4th day and were sacrificed for subinoculation purposes and for preparation of dried mouse-brain virus ; 1 mouse was paralysed on the 11th day and remained so until the 30th day, when it was destroyed. Of the 4 infant mice inoculated intraperitoneally with this suspension, 1 died on the 3rd and another on the 5th day, while the other 2 remained well. Of 6 adult mice inoculated with the Seitz-filtered supernate of the cervical and upper thoracic portions of the cord, 2 became paralysed on the 4th day and 1 on the 5th day ; these were sacrificed for subinoculation purposes ; one mouse of this group became paralysed on the 6th day and died on the 7th day, while another which became paralysed on the 6th day and 1 which became paralysed on the 8th day were still paralysed on the 30th day, when they were destroyed.

Of the 6 adult mice inoculated intracerebrally with the unfiltered supernate of the lower thoracic and lumbosacral portions of the cord, 1 became paralysed on the 3rd day, 1 was paralysed and 1 was sick on the 4th day and 2 were paralysed on the 5th day. All of these mice were sacrificed for subinoculation purposes or for the preparation of dried mouse-brain virus. The remaining mouse in this group became paralysed on the 6th day and died on the 11th day. All of the 4 infant mice inoculated intraperitoneally with this suspension were dead on the 3rd day. In the final group of adult mice inoculated intracerebrally with the Seitz-filtrate of this suspension, 1 mouse was paralysed on the 3rd day, 2 on the 5th day and 1 on the 6th day. All of these mice were sacrificed for subinoculation purposes or for preservation of mouse-brain virus ; the other 2 mice of the group remained well for 30 days.

Successful intracerebral transfers were carried out with 10 per cent suspensions of the brains of dead baby mice and of paralysed adult mice that had been sacrificed. Three lines of mouse brain to brain passage were made. The A line originated from the brain of a mouse, paralysed on the 4th day, belonging to a group inoculated with a Seitz-filtered suspension of the cervical and upper thoracic portions of the cord of monkey UR 22 ; and the B and C lines originated from the brains of mice, paralysed on the 4th and 5th day respectively, from a group inoculated with an unfiltered suspension of the lower thoracic and lumbo-

sacral portions of the cord of the same monkey. By the 2nd intracerebral passage there was 100 per cent mortality of the subinoculated groups of mice, and the average survival time of inoculated mice was 4·0 days in each group. By the 7th intracerebral passage of mouse-brain virus the average survival time of mice in lines A, B and C were 1·6, 2·2 and 2·8 days respectively.

Routine transfers were continued with line A. The average survival time of mice inoculated intracerebrally from the 9th to the 13th transfer was 2 days, and for those inoculated from the 14th to 17th transfer it was 1·5 days. From the 18th to 29th passages, transfers of unfiltered brain suspension were made intraperitoneally. The average survival time of mice thus inoculated was 2·1 days from the 18th to 21st transfer, 1·8 days from the 22nd to 25th, and 2·0 days from the 26th to the 29th. Subsequently subinoculations were made intraperitoneally, using 0·06 ml. quantities of the Seitz-filtered supernate of 10 per cent mouse-brain virus. The average survival time in mice thus inoculated was 1·7 days from the 30th to the 39th transfer. Since the 40th transfer the average survival time has remained very constant at about 1·3 days. This strain of virus is now (September 21, 1947) in its 108th passage in mice, having been maintained in refrigerated suspensions between mouse passages for various periods since June, 1946. The isolations of virus from the paralysed rhesus monkey, UR 22, were therefore from the cerebrospinal fluid and from various portions of the spinal cord.

Isolation from Taeniorhynchus *mosquitoes.*

Thirty-one days after UR 22 monkey was found paralysed a strain of MEV was isolated from a group of mosquitoes consisting of the *Taeniorhynchus* species. The mosquitoes were from a batch of 444 mosquitoes of various species which were caught at Zika, a forested area about 7 miles from Entebbe. The mosquito catch was one of a series of many routine catches which form part of our yellow fever investigation programme. The method employed in making such catches has been described elsewhere (Haddow, 1945). The mosquitoes were sorted into groups convenient for inoculation of animals. In the *Taeniorhynchus* group there were 3 *Taeniorhynchus* (*Coquillettidia*) *maculipennis* Theobald, 96 *T.* (*C.*) *fuscopennatus* Theo., 19 *T.* (*Mansonioides*) *africanus* Theo., and 1 *T.* (*M.*) *uniformis* Theo.

After sorting, the mosquitoes were inactivated by chilling in a refrigerator, and were then ground up with 3·0 ml. of physiological saline and centrifuged at low speed for 15 minutes. Mouse groups were inoculated with the unfiltered and Seitz-filtered supernates. One of the 6 mice inoculated with the unfiltered suspension was sick on the 9th day, but successful transfers were not made with the brain of this mouse. Of 6 mice inoculated with the Seitz-filtrate, 1 was found dead on the 12th day; all others remained well for 30 days. Transfers of mouse-brain suspensions were made from this dead mouse. Subinoculated groups of mice did not exhibit 100 per cent mortality till the 4th intracerebral passage. After the 5th transfer in 1 line of mouse-brain passage, mice were paralysed or dead within 24 hours; the average survival time by the 6th passage was 1 day with the use of unfiltered 10 per cent mouse-brain suspension. The average survival time of mice in passages 6 to 9, in which Seitz-filtered 10 per cent mouse-brain virus was passed intracerebrally, was 1·1 days. From the

10th to the 15th passage the subinoculations were made by the intraperitoneal route, and the average survival time was 1·9 days.

Thirty-five days after monkey UR 22 was found paralysed, an evening mosquito catch was made in and around the outdoor monkey run where UR 22 had been kept. The catch consisted of 216 *Taeniorhynchus* (*Coquillettidia*) *fuscopennatus* Theobald and 31 *T.* (*Mansonioides*) *africanus* Theo. After sorting and chilling the *T. fuscopennatus* were ground up in 5·0 ml., and the *T. africanus* in 2·0 ml., of physiological saline, and the suspensions were centrifuged. Groups of 6 mice were inoculated intracerebrally with Seitz-filtered and unfiltered suspensions. Nothing occurred in mice as a result of the inoculations with the suspension of *T. africanus*.

In the group inoculated with the unfiltered suspension of *T. fuscopennatus* 2 mice became paralysed on the 5th day and were sacrificed for subinoculation purposes and pathological studies. On the 6th day there were 3 additional paralysed mice, 2 of which died on the 9th day ; on the 21st day there was 1 moribund mouse and 1 survivor in the group. In the 2nd passage in mice subinoculated intracerebrally with the Seitz-filtered supernate of a 10 per cent mouse-brain suspension, there was 100 per cent mortality and the average survival time was 6·8 days. The average survival times of mice in the next 3 intracerebral transfers of Seitz-filtered suspensions were 7·6, 5, and 6·2 days. Subsequently transfers were made with filtered suspensions injected intraperitoneally in 0·06 ml. quantities. The average survival time in the next 5 transfers was 4·0 days ; from the 11th to the 18th transfer it was 2·4 days ; from the 18th to 29th it was 2·2 days ; and thereafter up to the 34th passage it was 2·0 days.

Isolation from the serum of a mongoose.

On September 2, 1946, a mongoose (*Ichneumia* sp.) was trapped on the compound of the Institute, and before being destroyed it was bled routinely for a yellow fever protection test. Fourteen-day-old mice were used in the test (Smithburn, 1945), and on the 2nd day it was found that all the mice inoculated with the mixture of mongoose serum and yellow fever virus were dead. With non-protective sera the incubation period in this test is at least 3 days, and deaths on the 2nd day are regarded as non-specific so far as yellow fever virus is concerned. In order to discover the reason for the " toxicity " of this serum, groups of adult mice were inoculated directly with a portion of the mongoose serum which had been kept in a refrigerator. MEV was isolated directly from the serum and successful serial intracerebral transfers were made.

As an interesting sideline to this isolation of MEV directly from the serum of the mongoose the following experiment was done: The brains and livers of the baby mice which had died on the 2nd day in the yellow fever protection test were ground up with yellow fever immune serum, centrifuged and incubated at 37° C. for 2 hours ; groups of mice were then inoculated. Six adult mice inoculated intracerebrally with the suspensions of brains of the 14-day-old mice were all dead on the 2nd day. MEV was identified as the infectious agent, and successful intracerebral transfers were made. From this experiment it may be seen that in animals infected with 2 viruses (in this case yellow fever and MEV) 1 virus may be isolated by the neutralization of the other by a specific immune serum.

Isolation from a rhesus monkey with fever but no paralysis.

On May 3, 1947, a rhesus monkey (MR 823), brought from the outdoor run to the laboratory, was bled for a yellow fever protection test and inoculated with a suspension of mosquitoes. This animal had been bled in March, 1947, for a yellow fever protection test but had not been submitted to any other experimental procedures. On arrival in the laboratory on May 3 it had a temperature of 40·0° C., but little attention was paid to this as monkeys often show an elevation of temperature after the chase necessary to capture them in the outdoor runs. However, its temperature was 40·3° C. on the following day, and in view of this fact 6 mice were inoculated intracerebrally with its serum obtained on May 3, which had been kept in a refrigerator overnight. One of the inoculated mice died within the first 24 hours, probably from trauma. Of the 5 remaining mice 1 appeared sick on the 3rd day and exhibited convulsive movements, and 1 was paralysed on the 4th day. These mice were sacrificed for subinoculation purposes. Another mouse became sick on the 6th day and was sacrificed for pathological studies. Of the remaining 2 mice, 1 became paralysed on the 8th day and was still paralysed on the 21st day, at which time there was 1 well survivor of the group. There was 100 per cent mortality from Mengo encephalomyelitis among the mice subinoculated intracerebrally with suspensions of the brains of sick and paralysed mice. The average survival time by the 3rd intracerebral passage of virus isolated from MR 823 was less than 24 hours when a Seitz-filtered 10 per cent mouse-brain suspension was used for the subinoculations.

This isolation of virus from a second rhesus monkey is in striking contrast with the original isolation from UR 22. In the first instance no virus was isolated from the blood, but lines were recovered from both the cerebrospinal fluid and the spinal cord, whereas in the second instance virus was recovered from the blood. However, the first monkey was past the febrile stage of its illness, if, in fact, it had had fever, when the materials were taken for inoculation. Rhesus 823 was in the febrile stage of its illness when the serum from which virus was isolated was obtained. This suggests that Mengo encephalomyelitis, in rhesus monkeys at least, is initially a systemic infection, and that central nervous system manifestations may or may not occur, depending on whether the virus localizes in the nervous system. However, it would appear that, by the time such localization occurs, the virus is no longer present in the blood.

Serological Specificity of MEV.

The techniques employed in serological specificity tests were based on methods used in studies of Bunyamwera (Smithburn, Haddow and Mahaffy, 1946), and Semliki Forest (Smithburn, Mahaffy and Haddow, 1944) viruses, and previously by Olitsky and Harford (1938) in experiments with equine encephalomyelitis viruses.

In preliminary experiments the preinoculation and convalescent sera of inoculated monkeys were tested for their neutralizing effect on MEV. The virus material for these tests was prepared by making a 10 per cent suspension of brains of infected mice in distilled water and centrifuging it at low speed for 15 to 20 minutes. Distilled water was used as diluent because we could not at this time be certain that any of our normal monkeys were non-immune. Tenfold dilutions of the supernate were made, using as diluent 10 per cent serum-saline

mixtures of each serum to be tested. To 0·5 ml. portions of each undiluted serum were added 0·25 ml. portions of the virus dilutions which had been made with the respective serum diluent. The mixtures were shaken, incubated for 1 hour and then inoculated intraperitoneally in 0·06 ml. quantities into mice in groups of 6.

In these initial experiments it was shown that when serial dilutions of MEV (UR 22 monkey strain) were mixed with preinoculation and other normal non-immune monkey sera, titres averaging $10^{-7.17}$ were obtained. Serum taken from Rhesus 692 thirty days after subinoculation with material from UR 22 (the original paralysed monkey) neutralized at least 31,700 LD_{50} of MEV, while serum taken from a monkey (621) 8 days after inoculation with mouse-brain MEV neutralized 282 LD_{50} of virus. It appeared therefore from these preliminary studies that an intraperitoneal neutralization test was satisfactory for studies of the identity of the virus, and for establishing the relationship of the various strains which were subsequently isolated.

TABLE I.—*Comparison of Titration Obtained when MEV is Mixed with Anti-Sera of Some of the Neurotropic Viruses Isolated in Uganda or with Yellow Fever Immune Serum.*

Serum.	Source.	Virus titre.*
Normal	Rhesus 690	$10^{-7.50}$
Normal	Rhesus 691	$10^{-6.87}$
Semliki forest virus immune	Rhesus 251	$10^{-6.51}$
Bunyamwera virus immune	Rabbit 20	$10^{-7.11}$
Ntaya virus immune	Rhesus 282	$10^{-6.50}$
Bwamba fever virus immune	*C. aethiops* 668	$10^{-7.00}$
Uganda S virus immune	Rhesus 922	$10^{-6.44}$
Yellow fever immune	Rhesus 265	$10^{-6.60}$
MEV immune	Rhesus 692	$< 10^{-2.00}$

* Here and in subsequent experiments titres refer to 50 per cent end-points as calculated by the method of Reed and Muench (1938).

Experiments using the technique described above were then done with yellow fever immune serum and anti-sera for some of the neurotropic viruses isolated in Uganda. From the results of these experiments, which are presented in Table I, it may be seen that the titres of MEV, when it is mixed with sera immune to yellow fever, Semliki forest (Smithburn, Mahaffy and Haddow, 1944), Bunyamwera (Smithburn, Haddow and Mahaffy, 1946), Ntaya, Bwamba fever (Smithburn, Mahaffy and Paul, 1941), or Uganda S virus are of the same order as titrations of this virus in normal control sera.

Further studies were made to find out whether MEV was identical with certain other neurotropic viruses. In these experiments, detailed in Table II, the serial dilutions of the virus suspensions were made with 10 per cent normal monkey serum or with saline, and end-point titrations of the immune-serum–virus mixtures were not carried out. The immune sera against the 5 heterologous viruses listed in Table II, Part 1, were prepared by repeated subcutaneous inoculations of the respective virus into rhesus monkeys. These sera were stored in the dry state and rehydrated before use. It may be seen from Table II,

Part 1, that while sera of rhesus monkeys which are immune either as a result of a natural infection (722) or inoculation (621) with MEV, can neutralize 317,000 or more LD_{50} of the virus, the sera of monkeys specifically hyperimmunized against western or eastern equine encephalomyelitis, Japanese B or St. Louis encephalitis, or West Nile virus (Smithburn, Hughes, Burke and Paul, 1940), neutralize less than 1000 LD_{50} of MEV. Specificity tests were also carried out with sera immune to the following: louping ill, supplied by Dr. D. G. ff. Edward, The Welcome Veterinary Research Station, lymphocytic choriomeningitis, Ilheus (Laemmert and Hughes, 1947) and Venezuelan (Kubes and Rios, 1939), equine encephalomyelitis, supplied by Dr. T. P. Hughes, staff member, International Health Division, The Rockefeller Foundation, poliomyelitis, supplied by Dr. H. D. Tonking, senior pathologist, Mauritius, Theiler's encephalomyelitis of mice, Far Eastern (Russian) tick-borne spring-summer encephalitis, and to a filterable infectious agent recently isolated at this laboratory, tentatively called MR 766 virus. The only available source of Theiler's encephalomyelitis immune serum was a pool of serum from (1) mice with spontaneous encephalomyelitis, and (2) mice which after inoculation developed encephalomyelitis clinically identical to Theiler's disease. Only mice which had been paralysed for over 20 days were bled for the immune serum pools. However, immunological studies with that virus are notoriously unsatisfactory, and the studies here made cannot be regarded as having differentiated Theiler's virus from MEV by protection tests. Nevertheless, there are many other characteristics of MEV which distinguish it clearly from Theiler's encephalomyelitis virus of mice (Theiler, 1937). Immune serum against Far Eastern (Russian) encephalitis virus was prepared by repeated subcutaneous inoculation of rabbits with a strain of virus kindly supplied by Dr. P. K. Olitsky, The Rockefeller Institute for Medical Research.

The results of these neutralization tests are presented in Parts 2 and 3 of Table II. From the preliminary neutralization experiments, it may be concluded that MEV is not identical with any of the neurotropic viruses against the anti-sera of which it has been tested. Complete cross-neutralization tests to study the antigenic relationship of MEV to other neutrotropic viruses are being made but have not as yet been completed.

A relationship between MEV and the virus of encephalomyocarditis (Schmidt, 1948) has been demonstrated. This work has been done independently by Dr. J. Warren at the Army Medical Center, Washington, and one of us (G. W. A. D.) at the Laboratories of the International Health Division of the Rockefeller Foundation, New York.

Identity of Strains of MEV.

By experiments using the technique described above, it was shown that convalescent serum from a monkey (MR 692) which had been inoculated with UR 22 monkey virus neutralized at least 10,000 LD_{50} of the virus strains isolated from the *T. fuscopennatus*, the *Taeniorhynchus* spp. and the mongoose. The final proof of the identity of these 3 strains with the UR 22 monkey strain was established by the following cross-neutralization experiment:

Sera from 3 rabbits (No. 169, 170 and 171) were each tested for neutralizing antibody against the UR 22 monkey strain, the *T. fuscopennatus* strains, and the *Taeniorhynchus* spp. strain of virus. The titres obtained demonstrated

TABLE II.—*Intraperitoneal Neutralization Tests with MEV and Anti-Sera of Some Neurotropic Viruses.*

No.	Serum.	Source.	Survival ratio* of mice inoculated with serum plus virus dilution. 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Virus titre.**	LD_{50} of virus neutralized.
1	Normal	Rhesus 621	0/6	0/6	0/6	0/6	0/6	0/6	6/6	$10^{-6.50}$	0
	W. E. E (*a*) hyperimmune	Rhesus 4275	..	0/6	0/6	..	..	..	..	$10^{-3.50}$ or >	< 1000
	E. E. E. (*b*) hyperimmune	Rhesus 4274	..	0/6	0/6	..	..	..	..	$10^{-3.50}$ or >	< 1000
	St. L. (*c*) hyperimmune	Rhesus 4287	..	0/6	0/6	..	..	..	..	$10^{-3.50}$ or >	< 1000
	Jap. B. (*d*) hyperimmune	Rhesus 4273	..	0/6	0/6	..	..	..	..	$10^{-3.50}$ or >	< 1000
	West Nile hyperimmune	Rhesus 4269	..	0/6	0/6	..	..	..	..	$10^{-3.50}$ or >	< 1000
	M. E. (*e*) immune	Rhesus 621	5/5	6/6	5/5	..	..	..	..	$10^{-1.00}$ or <	> 317,000
	M. E. (*e*) natural immune	Rhesus 722	5/6	6/6	5/5	..	..	..	..	$10^{-1.00}$ or <	> 317,000
2	Normal	Rhesus 431	..	0/6	0/6	0/6	0/6	1/6	6/6	$10^{-6.40}$ or >	0
	Louping ill immune	Sheep 11	..	0/6	0/6	1/6	..	..	..	$10^{-4.40}$ or >	< 79
	L. C. M. (*f*) immune	Rabbit 273	..	0/6	0/6	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	Ileus immune	Rhesus 4965	..	0/6	0/6	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	V. E. (*g*) immune	Rabbit 314	..	0/6	0/6	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	Poliomyelitis immune	Human pool	..	0/6	0/6	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	Mouse encephalomyelitis immune	Mouse pool	..	0/6	0/6	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	MR 766 (*h*) immune	Rhesus 766	..	0/6	0/5	0/6	..	..	..	$10^{-4.50}$ or >	< 79
	M. E. (*e*) immune	Human convalescent	..	3/6	6/6	6/6	..	..	..	$10^{-2.50}$ or <	> 7900
3	Normal	Rhesus 690	..	0/6	0/6	0/6	0/6	0/6	2/6	$10^{-7.25}$ or >	0
	Far eastern (Russian) immune	Rabbit 346	..	..	..	0/6	1/6	2/6	..	$10^{-6.12}$ or >	14 or >
	M. E. (*e*) immune	Rhesus 621	..	6/6	6/6	6/6	..	..	..	$10^{-2.00}$ or <	> 178,000

* The numerator indicates the number of survivors and the denominator the number of inoculated mice.
** See footnote, Table I.
(*a*) Western equine encephalomyelitis.
(*b*) Eastern equine encephalomyelitis.
(*c*) St. Louis encephalitis.
(*d*) Japanese B encephalitis.
(*e*) Mengo encephalomyelitis.
(*f*) Lymphocytic choriomeningitis.
(*g*) Venezuelan equine encephalomyelitis.
(*h*) A recently isolated filterable neurotropic agent.

that none of the rabbits had neutralizing antibodies to any of these strains of virus. Rabbit 169 was then hyperimmunized against the UR 22 strain, Rabbit 170 against the *T. fuscopennatus* strain, and Rabbit 171 against the strain isolated from the mixed batch of *Taeniorhynchus* spp. This was accomplished by inoculating the rabbits at 3-day intervals with 1 ml., 1 ml., and 0·75 ml. of 10 per cent mouse-brain suspensions of one or other of the 3 strains of virus. Ten days after the last injections of virus the rabbits were bled and, by means of the intraperitoneal protection test already described, a cross-neutralization test was made. In the results of this experiment as presented in Table III, the LD_{50} of virus neutralized in each case is calculated from the titre of each virus mixed with the homologous normal serum only. The titres of the heterologous normal sera were of the same order as those presented.

The identity of the mongoose strain of virus and the UR 22 monkey strain was established by a second similar cross-neutralization. Rabbit 168 was hyperimmunized by repeated subcutaneous injections of mouse-brain passage virus which originally had been isolated from the mongoose serum. Virus-serum mixtures were prepared as described above, and groups of mice were thereafter inoculated with the mixtures. It was shown that the convalescent serum of Rabbit 168 neutralized more than 1780 LD_{50} of both the UR 22 monkey strain and the mongoose strain of virus, and that UR 22 monkey strain immune serum neutralized more than 31,700 LD_{50} of the mongoose strain of virus.

A similar type of experiment was done with the MR 823 strain of virus. The MR 823 mouse-brain virus was completely neutralized by MEV (UR 22 monkey strain) immune serum, but not by non-immune serum. Inactivated acute-phase and convalescent sera of MR 823 were tested against this virus and

TABLE III.—*Results of Cross-Neutralization Test with UR 22 Monkey Strain,* T. fuscopennatus *Strain and* Taeniorhynchus *spp. Strains of Virus.*

Virus strain.	Rabbit serum.	Effective virus titre.*	LD_{50} of virus neutralized.
UR 22 monkey.	Normal UR 22 monkey strain (169 preinoculation)	$10^{-6.40}$	0
	Immune UR 22 monkey strain (169 convalescent)	$< 10^{-3.00}$	> 2500
	Immune *T. fuscopennatus* strain (170 convalescent)	$< 10^{-3.00}$	> 2500
	Immune *Taeniorhynchus* spp. strain (171 convalescent)	$< 10^{-3.00}$	> 2500
T. fusco-pennatus	Normal *T. fuscoppenatus* strain (170 preinoculation)	$10^{-6.36}$	0
	Immune *T. fuscopennatus* strain (170 convalescent)	$< 10^{-2}$	$> 23,200$
	Immune UR 22 monkey strain (169 convalescent)	$< 10^{-2}$	$> 23,200$
	Immune *Taeniorhynchus* spp. strain (171 convalescent)	$< 10^{-2}$	$> 23,200$
Taenio-rhynchus spp.	Normal *Taeniorhynchus* spp. strain (171 preinoculation)	$10^{-6.44}$	0
	Immune *Taeniorhynchus* spp. strain (171 convalescent)	$< 10^{-2}$	$> 25,500$
	Immune UR 22 monkey strain (169 convalescent)	$< 10^{-2}$	$> 25,500$
	Immune *T. fuscopennatus* strain (170 convalescent)	$< 10^{-2}$	$> 25,500$

* See footnote Table I.

against UR 22 monkey strain of MEV. At the time when this experiment was made the marked stability of MEV was not known. It is conceivable that inactivated acute-phase serum from MR 823 contained active virus. The acute-phase serum had no protective power against either the MR 823 or UR 22 monkey strain of MEV, while the convalescent serum of rhesus 823 neutralized more than 10,000 LD_{50} of both. Moreover, the MR 823 monkey virus was again completely neutralized by MEV (UR 22 monkey strain) immune serum. This virus was therefore proved to be another strain of the Mengo encephalomyelitis virus.

The serological identity of a virus isolated from a human case of encephalitis with that of other strains of Mengo encephalomyelitis has been reported elsewhere (Dick, Best, Haddow and Smithburn, 1948).

Immunity to the Virus.

Immunity to MEV has been demonstrated in man. No specific surveys have been made for the purpose of studying the incidence of immunity to this

virus among animals, but a number of sera from unused monkeys in the outdoor runs of the laboratory, from wild monkeys and from mongooses have been tested for the presence of neutralizing antibodies. In testing these sera 0·25 ml. portions of a 1 in 100 dilution of mouse-brain virus were added to 0·5 ml. of the serum under test. The virus-serum mixtures were allowed to stand at room temperature for periods of 20 to 30 minutes before inoculation into mice. The virus suspension employed in the test was titrated from 10^{-2} to 10^{-6} against undiluted normal serum. An immune control was set up in which immune human or monkey serum was diluted from 1 in 4 to 1 in 256, and 0·25 ml. of 1 in 100 virus was added to undiluted, and to each dilution of, immune serum. The test dose of virus was usually in the region of 1000 LD_{50} per mouse. In all tests early-passage virus was employed. Mice were observed for 10 days. If 5 or more mice survived, the serum was regarded as protective, if 2 or less mice survived it was regarded as non-protective. If more than 2 but less than 5 mice survived, the test was considered inconclusive and the serum was retested, usually against 2 or more dilutions of virus. In a few of the cases in which serum-virus mixtures produced 100 per cent mortality, the mice showed a survival time longer than the average; these sera were also retested against high dilutions of virus. In all cases the occasional serum which gave an inconclusive result was negative on retest. All sera in which a clear-cut positive result was obtained remained positive on retest.

Tests made on sera from 23 rhesus monkeys from the outdoor runs showed that 8 (35 per cent) were immune to MEV. In none of these monkeys was any paralysis recorded. In the case of 2 of them preinfection sera were available for comparison with the later sera found to contain antibody. The results of the protection tests indicated that immunity in these 2 monkeys had been naturally acquired on the institute compound within the past year. As in the case of rhesus monkeys, natural infection has occurred among the normal stock of *Cercopithecus aethiops centralis* Neumann monkeys. Of 6 unused *C. aethiops* monkeys 1 was found to be immune, but to date none of them has been found paralysed. Monkeys which have survived experimental inoculations of MEV, or have experienced natural infections with this virus (Table II) have developed a high degree of immunity. Sera from 18 wild monkeys from western and central Uganda, which had been obtained for yellow fever protection tests, were tested against MEV, but none of them neutralized this virus.

No tests have as yet been made with the sera of other wild animals except those of 4 mongooses (of unidentified species) trapped in the compound of the institute. One of these neutralized MEV.

DISCUSSION.

The first isolation of MEV was made in June, 1946, from the cerebrospinal fluid and the central nervous system of a spontaneously paralysed afebrile rhesus monkey (UR 22) which belonged to the reserve stock of unused rhesus monkeys. These monkeys are housed in the outdoor runs of the laboratory in the institute compound. In the following month strains of virus were isolated from a mixed group of *Taeniorhynchus* spp. caught in a forested area 7 miles from the laboratory and from *T. fuscopennatus* caught in and around the monkey run in which UR 22 had been housed. In September, 1946, a further strain was isolated from a

mongoose which was trapped in the compound of the institute. In November, 1946, a human strain of MEV was isolated from the blood of a man with an acute febrile illness characterized by signs and symptoms referable to the central nervous system. This virus was not encountered again until May, 1947, when it was isolated from the blood of a rhesus monkey (MR 823) which had a febrile illness but which at no time showed any signs of paralysis. This monkey, like UR 22, was housed in the outdoor runs of the laboratory where unused rhesus monkeys are kept. Evidence has been presented which shows that the 6 viruses isolated are identical strains of MEV.

The isolation of a strain of MEV from *Taeniorhynchus* (*Coquillettidia*) *fuscopennatus* Theobald mosquitoes and from a mixed group of *Taeniorhynchus* mosquitoes (consisting mainly of *T.* (*C.*) *fuscopennatus*) is circumstantial evidence that *T.* (*C.*) *fuscopennatus* may be a natural vector. There is no evidence of other biting arthropods being responsible for transmission. Experiments on the mode of transmission are planned but have not yet been made.

The isolation of a strain of the virus from a mongoose (*Ichneumia* sp.) and the demonstration of neutralizing antibodies to this virus in the serum of another mongoose suggests that these animals may possibly act as hosts of this virus.

MEV induces a high degree of immunity in animals which survive inoculation with it. A similar degree of immunity has been demonstrated in 8 of 23 unused rhesus monkeys and in 1 of 6 *C. aethiops* monkeys from the outdoor monkey runs in the institute compound, presumably as a result of natural infection. In none of these monkeys was paralysis recorded. Paralysis in monkeys naturally infected would appear to be rare. The occurrence of a considerable number of non-paralytic infections produced by MEV among monkeys in the open runs of the laboratory is similar to what is found in humans infected with poliomyelitis. Studies of the antigenic relationship, if any, between the virus of poliomyelitis and that of Mengo encephalomyelitis have been planned.

Only a limited study of immunity to this virus among humans and wild animals has been made, but the demonstration of immunity among humans in the Western Province of Uganda is evidence that the activities of Mengo encephalomyelitis virus are not restricted to the district of Mengo (Uganda).

The possible relationship of MEV to other neurotropic viruses has not yet been studied completely. It would appear, however, that MEV is not identical with any of the 19 neurotropic viruses against the anti-sera of which it has been tested. On the basis of the pathogenicity of MEV for experimental animals, the pathological lesions which it induced and its physical properties (Dick, 1948), it would appear to be different from virus III, virus B, and the pseudorabies, Australian X, Anopheles A and B (Rocca-Garcia, 1944), and Wyeomyia viruses. No anti-sera of these latter viruses have been available for neutralization tests.

SUMMARY.

1. Filterable viruses have been isolated from a paralysed rhesus monkey (UR 22), which had not been used for experimental purposes, from mosquitoes of the species (*Taeniorhynchus* (*Coquillettidia*) *fuscopennatus* Theo., and from a mixed lot of *Taeniorhynchus* spp.), from a mongoose (*Ichneumia* sp.) and, a year after the first isolation, from an unused rhesus monkey which had pyrexia but no paralysis.

2. Evidence is presented which shows that the above-mentioned viruses are all strains of the same virus.

3. The virus has been called Mengo encephalomyelitis virus (MEV) because it was isolated in Mengo District in Buganda, Uganda, and because it can produce lesions in the brains and spinal cords of infected animals and encephalitis symptoms in man.

4. Neutralizing antibodies to this virus have been demonstrated not only in sera of convalescent inoculated animals but also in the sera of 8 of 23 unused rhesus monkeys, 1 of 6 unused *C. aethiops* monkeys captive in the outside runs of the laboratory, and 1 of 4 mongooses trapped in the institute compound.

5. MEV has been found to be immunologically distinct from each of the other 19 neurotropic viruses with which it has been compared. In its physical and pathogenic properties it appears to be different also from certain other neurotropic viruses with which it has not yet been possible to compare it.

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MENGO ENCEPHALOMYELITIS VIRUS: PATHOGENICITY FOR ANIMALS AND PHYSICAL PROPERTIES.

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In a recent communication the isolation of Mengo encephalomyelitis virus from 2 rhesus monkeys, a mongoose, a batch of *Taeniorhynchus fuscopennatus* mosquitoes and a mixed batch of mosquitoes of the genus *Taeniorhynchus* was presented, and preliminary studies of the immunological properties of the virus were described (Dick, Smithburn and Haddow, 1948). The isolation of a strain of the virus from a human was reported by Dick, Best, Haddow and Smithburn (1948). The purpose of the present paper is to discuss the pathogenic properties of the virus for laboratory animals, the histological lesions which it induces and the physical properties of the virus.

PATHOGENIC PROPERTIES OF THE VIRUS.

Unless otherwise stated the strain of virus isolated from the paralysed rhesus monkey, UR 22, (Dick, Smithburn and Haddow, 1948) was employed in all experiments.

In Mice.

The human, monkey, mongoose and mosquito strains of Mengo encephalomyelitis virus (MEV) were first demonstrated by virtue of their pathogenicity for mice. Apart from some slight variations in the incubation period and average survival time of mice inoculated with early-passage virus, the behaviour of all 6 strains of the virus in mice has been identical. All the strains caused 100 per cent mortality in inoculated mice by the 2nd intracerebral passage, except the one isolated from the mixed group of *Taeniorhynchus* mosquitoes. In the latter case 100 per cent mortality occurred by 3rd and 4th transfer in 2 lines of passage and by the 6th transfer in the line with which serial passages were continued. In Table I is presented a comparison of the average time of survival in the early passages in which mice were inoculated intracerebrally. The mice employed in all experiments are descended from the original stock of Swiss White mice from Carworth Farms near New York City. Mice used for experiments were between 35 and 42 days old unless otherwise stated. A group of mice consists of 6, which, under routine, are caged together on weaning at 21 days of age.

In the early passages in adult mice the first clinical evidence of illness was usually paralysis of one or more limbs. As a rule both hind limbs exhibited a flaccid paralysis. If the mouse survived the paralysis of the hind limbs for more than 4 days, the front limbs usually then became paralysed. In some of the early passages, and with small quantities of virus in later passages, mice occasionally remained paralysed throughout the period of observation of 21 to 30 days. In general, however, after the 2nd mouse passage, prostration and

TABLE I.—*Comparison of Average Survival Time in the First Ten Passages of Mice Inoculated with Human, Rhesus, Mongoose, Mixed* Taeniorhynchus, *and* T. fuscopennatus *Strains of MEV.*

Number of passages in mice.	Average survival time in days of mice inoculated intracerebrally with 10 per cent Seitz-filtered mousebrain suspension of strain from					
	Serum.			Mosquitoes.		Spinal cord.
	Human.	Mongoose.	Febrile Rhesus 823.	*Taeniorhynchus fuscopennatus.*	*Taeniorhynchus* spp.	Paralysed Rhesus UR 22.‡
2	1·2	1	1·4	5	— 3 survivors	5
3	1·4	1	*	6·8	— 5 ,, †	3·5
4	1·0	1	*	4·4	— 2 ,, †	3·5
5	1·4	*	*	3·8	3 *	3·0
6	1·2	*	*	4·6	4·2	4·3
7	1·0	*	*	2·2	2·6	2·8
8	1·4	*	*	2·6	1·4	2·2
9	1·0	*	*	*	1·5	1·6
10	1·0	*	*	*	*	2·0

* Transfer made by intraperitoneal route.
† Transfer made with 10 per cent Seitz-filtered live suspension.
‡ Line of virus isolated from a suspension of lower thoracic and lumbar cord.

death supervened rapidly after the onset of symptoms. Following intracerebral inoculation of relatively large doses of early-passage virus, the symptoms first appear 48 to 56 hours after inoculation, and all mice may be dead within 72 hours. Paralysis in these mice is sometimes, but not always, preceded by a rough appearance of the fur. With small doses of virus (about 100 LD_{50}), sufficient to cause death of all mice, the incubation period is sometimes prolonged to 5, 6, or 7 days, and death, although usually occurring within 2 to 3 days after the onset of symptoms, may be delayed until the 10th to the 16th day. Mice which have been paralysed for a few days are incontinent, but the tail escapes paralysis. With later-passage virus death usually occurs within 24 to 48 hours after inoculation and without any preceding evidence of paralysis or sickness.

Intraperitoneal inoculations produce a similar sequence of events. When early-passage virus is used, the incubation period is about 3 days if relatively large doses of virus are given, and between 5 and 14 days if high dilutions of virus are employed. With later-passage virus mice become paralysed and die within 48 hours. By intranasal or subcutaneous inoculation of virus the incubation period is prolonged by 24 to 48 hours over that following intraperitoneal inoculation. Mice inoculated subcutaneously become paralysed and die a little earlier than mice inoculated intranasally.

The efficiency of various routes of inoculation was tested with both early and late-passage virus. For this purpose 10 per cent suspensions of infective mouse-brain tissue were prepared and centrifuged at low speed for 20 minutes. Serial tenfold dilutions of the supernates were made, using 10 per cent normal serum-saline as diluent. The term 10 per cent serum-saline indicates a mixture of 10 volumes of whole normal monkey serum and 90 volumes of 0·86 per cent sodium chloride. The results of these experiments are presented in Table II.

Mice 4 to 6 days old die within 24 to 48 hours after intraperitoneal inoculation with 0·06 ml. quantities of 10 per cent mouse-brain passage virus. There is no significant difference in the susceptibility of mice 3 to 12 weeks of age to the extraneural inoculation of this virus, but the average survival time in old mice

inoculated with 10 per cent mouse-brain virus is twice as long as that of 21-day-old mice inoculated with the same suspension.

Distribution of virus in tissues of inoculated mice.

Studies were made to determine the distribution and relative concentration of MEV in the blood stream and viscera of inoculated mice. One mouse of the 15th passage was bled from the heart 48 hours after intraperitoneal inoculation

TABLE II.—*Comparison of Titrations in Mice by Various Routes of Inoculation of 3rd and 93rd Passage Mouse-Brain Virus.*

Passage number.	Route of inoculation.	Volume inoculated (ml.).	Virus dilution.	Mortality ratio.*	Virus titre.†
3	Intracerebral	0·03	10^{-5}	6/6	$10^{-6.64}$
			10^{-6}	5/6	
			10^{-7}	2/6	
93	Intracerebral	0·03	10^{-6}	6/6	$10^{-8.50}$ or >
			10^{-7}	6/6	
			10^{-8}	6/6	
3	Intraperitoneal	0·06	10^{-5}	6/6	$10^{-7.25}$ or >
			10^{-6}	6/6	
			10^{-7}	4/6	
93	Intraperitoneal	0·06	10^{-5}	6/6	$10^{-7.24}$
			10^{-6}	5/6	
			10^{-7}	4/6	
			10^{-8}	1/6	
93	Subcutaneous	0·06	10^{-6}	6/6	$10^{-8.00}$ or >
			10^{-7}	6/6	
			10^{-8}	3/6	
93	Intranasal	0·03	10^{-3}	6/6	$10^{-5.30}$
			10^{-4}	5/6	
			10^{-5}	3/6	
			10^{-6}	2/6	
			10^{-7}	1/6	
			10^{-8}	0/6	

* Here and in subsequent tables showing mortality ratios, the numerator indicates the number of mice which died, and the denominator indicates the number inoculated.

† All titres in this and subsequent experiments have been calculated by the 50 per cent end-point method of Reed and Muench (1938).

and before the appearance of any signs of illness. A group of mice was immediately inoculated intraperitoneally with the blood of this animal, each mouse receiving 0·06 ml. The remainder of the specimen was used to make serial dilutions of the blood, with normal saline as diluent. The titre of the virus in the blood stream was $10^{-4.07}$, showing that this virus is present in high concentration in the blood stream of inoculated mice before the onset of objective signs.

In another experiment 2 paralysed mice of the 34th passage were sacrificed. Their brains, spinal cords, spleen, kidneys, livers, hearts and lungs were removed aseptically; clean instruments were used for each tissue from each mouse. The entire organs with the exception of the liver, from which portions of all lobes were taken, were weighed to the nearest mg. and suspended in 10 per cent serum-saline to make 10 per cent suspensions. The preparations were centri-

fuged simultaneously at low speed for 10 minutes, and the supernates were passed through Seitz E. K. pads. Groups of 6 mice were inoculated intracerebrally with serial decimal dilutions of each tissue suspension. The results of this experiment are presented in Table III.

TABLE III.—*Results of Intracerebral Titrations of Tissues of Mice Inoculated with MEV Showing Distribution of Virus 48 Hours After Intraperitoneal Inoculation.*

Tissue.	Titre of virus.
Brain . .	$10^{-8.25}$ or >
Spinal cord .	$10^{-8.50}$ or >
Spleen . .	$10^{-4.37}$
Kidney . .	$10^{-3.38}$
Liver . .	$10^{-2.15}$
Heart . .	$10^{-3.60}$
Lung . .	$10^{-3.12}$

The high concentration of virus in the brain and cord, as compared with other organs, shows how strictly neurotropic this virus is. The presence of virus in relatively low concentrations in organs other than the central nervous system could be accounted for by the virus content of the blood.

Multiplication of virus in the brains of mice.

The rate of multiplication of the virus in the brains of mice and the quantity of virus circulating in the blood after intranasal inoculation were studied. In the first experiment 18 mice were each inoculated intranasally with 0.03 ml. of the unfiltered supernate of a 10 per cent suspension of 93rd-passage mouse-brain virus. After 30 minutes, 1, 3, 4, 8, 11, 24, 48 and 72 hours, 2 mice were anaesthetized and bled from the heart into syringes which had been rinsed out with heparin. The mice were then sacrificed and their brains were removed. The blood specimens and 10 per cent suspensions of the brains of each pair were separately pooled and centrifuged for 15 minutes at low speed. Groups of mice were inoculated with undiluted plasma and with various dilutions of the unfiltered supernates of the brain suspensions. Results of the experiments are shown in Expt. No. 1, Table IV.

TABLE IV.—*Results of Titrations of Virus Content in the Blood and Brains of Mice Inoculated Intranasally.*

Expt. No.	Material tested.	Titre after hours. ½	1	2	3	4	6	8	11	24	48	72
1	Plasma .	$1^{-1.00}$	$1^{-1.50}$ or >	—†	$1^{-1.50}$ or >	$1^{-1.50}$ or >	—	$1^{-1.50}$ or >	$1^{-1.50}$ or >	$1^{-1.50}$ or >	$1^{-1.50}$ or >	$1^{-1.50}$ or >
	Brain .	0	0	—	0	$10^{-1.38}$	—	$10^{-2.40}$	$10^{-2.00}$	$10^{-3.60}$	$10^{-6.50}$	$10^{-6.30}$
2	Serum .	$1^{-1.30}$	0	$1^{-1.00}$	—	$10^{-4.40}$ or >	$10^{-4.25}$ or >	—	—	$10^{-3.40}$	$10^{-2.75}$	—
	Brain .	0	0	0	—	0	$10^{-1.60}$	—	—	$10^{-3.75}$	$10^{-6.36}$	—

† Indicates no test made.

It may be seen that virus was present in the circulation within half an hour of intranasal instillation of virus, and could thereafter be demonstrated in the blood up to the 72nd hour. On the other hand, no virus could be demonstrated in the brains of mice until the 4th hour following inoculation, after which time rapid multiplication of virus occurred.

In the second experiment (Table IV) 42 mice were inoculated intranasally with a similar dose of virus, and 6 of these mice were sacrificed at 30 minutes, 2, 4, 6, 24 and 48 hours. At each time interval 6 mice were bled from the heart, the blood specimens were separately pooled and the serum was tested in various dilutions in separate groups of mice. The mice of each group were exsanguinated after bleeding from the heart; the brains were removed, pooled, and suspended in saline to make 10 per cent suspensions, and the supernates were titrated in groups of mice. In this experiment virus was again demonstrated in the serum within 30 minutes after intranasal inoculation. The virus content of the blood stream rose rapidly to its maximum titre in about 4 to 6 hours. No virus was demonstrated in the brain until 6 hours after inoculation, but from then on multiplication of virus took place rapidly there. Further experiments are required before it can be determined whether the rising titre of virus in the blood is due to absorption of virus from the nasopharynx or elsewhere, prior to its localization and multiplication in the central nervous system.

Infection by contact.

The possibility of infection by contact was investigated in several experiments. Six mice inoculated with MEV were added to a box of marked normal mice. When the inoculated mice became paralysed a transfer was made, and the six marked mice were caged with a group of freshly inoculated mice. The experiments were carried out with both male and female groups. Of a total of 18 normal female mice exposed to 204 infected female mice in three experiments, 2 became infected (18 per cent); and of 18 normal male mice exposed to 198 infected male mice, 13 became infected (72 per cent). This higher incidence of contact infections among males exposed to infected males suggests that the transmission of virus might be caused by fighting and cannibalism, which were in greater evidence among male than among female groups. In another experiment 6 normal female mice were caged with 6 infected males and *vice versa*. In the box containing the female contacts no infection took place; of the male contacts, 1 became infected. In all these experiments there was overcrowding of mice, since 12 mice were caged in boxes designed to house 6 mice only. When there is no overcrowding, contact infections do not occur. For example, in one experiment, in which mice of both sexes were used, 6 infected mice were added to a spacious box which contained 24 normal mice. No contact infections occurred, nor under these conditions was there any evidence of fighting or cannibalism. A final experiment was done to determine whether contact infections were likely to occur among mice in groups of 6 and thus possibly influence endpoint titres. Twelve groups of 6 mice each were inoculated in the following way. In each of 2 groups, 1 mouse was inoculated and the other 5 were left as contacts; in the next 2 groups 2 mice were inoculated and 4 served as contacts, and so on, until in the final groups 5 mice were inoculated and 1 served as contact. In the case of the boxes with 3 inoculated mice and 3 contacts, 4 groups of mice

were used. There were no contact infections in these groups; therefore, under normal conditions of experiments contact infections do not take place.

In Guinea-pigs.

Guinea-pigs are susceptible to the inoculation of MEV by the intracerebral, subcutaneous, intraperitoneal and intradermal routes. Juvenile guinea-pigs are more susceptible than older ones. Two young guinea-pigs (142 and 143) were inoculated intracerebrally with 0·15 ml. of a pool of the unfiltered supernate of suspensions of brain and cord of the paralysed rhesus monkey (UR 22) from which MEV was first isolated (Dick, Smithburn and Haddow, 1948). Neither of these animals showed any objective signs as a result of the inoculation. They had fever on the 3rd to 5th and 4th to 7th days respectively after inoculation. Circulating virus (identified as MEV) was demonstrated in the serum of No. 142 on the 3rd to 5th days. This animal died after cardiac puncture on the 9th day. Serum taken from the other guinea-pig (143) on the 24th day after inoculation contained antibody which neutralized MEV.

Two guinea-pigs (173 and 174) were inoculated intracerebrally with 0·1 ml. of serum from rhesus monkey 739, which, as a result of inoculation, was circulating the human strain of the virus. The two animals were dead within 24 hours and 48 hours respectively. The virus was passed serially to two other guinea-pigs (175 and 176), which were found dead within 24 hours. In all cases MEV was identified in the brains of the dead guinea-pigs. A third intracerebral passage of this line of virus was made by the subinoculation of guinea-pig 177, which died on the 3rd day without having exhibited any paralysis.

Of 5 guinea-pigs inoculated intracerebrally, intraperitoneally or subcutaneously with mouse-brain passage MEV, 1 which was inoculated subcutaneously died on the 8th day after inoculation. The others survived or were sacrificed for subinoculations. All of these animals exhibited fever for varying periods up to the 5th day after inoculation. Circulating virus was present for the first 3 days in the sera of those animals which were tested. Antibody was demonstrated in the sera of all animals which survived, and virus identified as that of Mengo encephalomyelitis was reisolated from the brains of all sacrificed guinea-pigs. In none of these animals was any paralysis seen.

In only 3 of the 22 inoculated guinea-pigs was paralysis observed. Two of the guinea-pigs which became paralysed were inoculated intracerebrally with suspensions of 2nd guinea-pig–brain passage virus. Both developed paralysis of the hind limbs on the 2nd day after inoculation; one of them (148) died on the 2nd day. A suspension of the brain of the latter was inoculated intracerebrally into guinea-pig 341. This animal developed fever and weakness of the hind quarters on the 4th day, and complete paralysis of the hind limbs set in on the 5th day. By the 8th day there was also paralysis of the left fore limb. The animal was sacrificed on the 9th day for histological material.

In Rabbits.

The pathogenicity of MEV has been studied in relatively few of these animals. For the purpose of studying the relationship of certain of the strains of this virus, rabbits were inoculated subcutaneously at 3-day intervals with massive

doses (over 33,300,000 mouse-intracerebral LD_{50}) of virus. None of these rabbits showed any clinical reactions, but all developed highly protective sera as the result of the inoculations.

Rabbits would appear to be insusceptible to the intracerebral inoculation of mouse-brain passage virus. While virus was recovered up to the 3rd day after inoculation from the brains of some inoculated rabbits, there was no evidence of multiplication of virus in their brains, and passage of rabbit brain to rabbit did not produce encephalitis in the subinoculated animal.

In Monkeys.

Rhesus monkeys.

Although MEV was first isolated from the brain and cord of a paralysed rhesus monkey (UR 22), paralysis must be a rare manifestation of activity of this virus in rhesus monkeys. Only 2 of 9 rhesus monkeys inoculated with MEV exhibited paralysis. Furthermore, Rhesus 823, a naturally infected rhesus monkey, from which a strain of virus was isolated, showed no abnormality apart from pyrexia, and 8 rhesus monkeys from the outdoor runs of the laboratory found to be immune as a result of naturally acquired infections with MEV had no record of paralysis.

Both of the rhesus monkeys (692 and 600) in which paralysis was observed were inoculated with monkey-passage virus. The inoculation used for the former consisted of a pool of the unfiltered supernates of portions of the cortex, midbrain, pons, cerebellum, medulla and cord of the original paralysed rhesus monkey (UR 22); 0·5 ml. of this pool was injected intracerebrally and 2 ml. subcutaneously. Rhesus 692 had fever (104·4° F.) on the 3rd day. It was bled daily for 10 days after inoculation, and the serum from the 4th to 7th days' bleedings produced paralysis and 100 per cent mortality when inoculated intracerebrally into groups of mice. The mortality ratio in mice inoculated with 3rd-day serum was 2/6. On the 9th day this monkey had slight weakness of the lower limbs, and there was a suggestion of incoordination. By the 10th day, however, it again appeared normal, and serum taken at that time contained antibody to MEV.

The other rhesus monkey (600) in which there was evidence of paralysis was inoculated intracerebrally with 0·1 ml. of 8th-day serum of Rhesus 739, from which the human strain of MEV had been recovered. Rhesus 600 had pyrexia from the 2nd to the 8th day after inoculation, and virus was demonstrated in the serum on the 2nd day; no other tests were made for circulating virus. From the 4th to 6th days Rhesus 600 had anorexia, and it showed some weakness of the left leg on the 7th day. It made a complete recovery and developed immunity to MEV.

With the exception of these 2 monkeys (692 and 600) and Rhesus 739, all rhesus monkeys were inoculated with mouse-brain virus, and further studies of rhesus monkey to rhesus monkey passage of MEV are required.

Rhesus 739 was inoculated intracerebrally with 0·1 ml. of 2nd-day acute-phase serum from the human patient. On the 7th day after inoculation it had a slight elevation of temperature (103·8° F.), and on the following day a temperature of 105° F. It was bled on the 8th day, and its serum was subinoculated into rhesus monkey 600, which has already been discussed. Rhesus 739 con-

tinued to show fever for 5 days, but no other manifestations were noted. Mice inoculated with the 8th-day serum of Rhesus 739 showed 100 per cent mortality (6/6). Convalescent serum from this latter monkey contained antibody to MEV in high titre.

Six rhesus monkeys were inoculated with mouse-brain passage MEV by the following routes: intracerebral (621), intracerebral and subcutaneous (838), intraneural (690), intravenous (826), intramuscular (829), and by subcutaneous inoculation of virus following an intracerebral inoculation of soluble starch (764). All of these monkeys had fever for varying periods from the 1st to 7th day after inoculation. Apart from Rhesus 838, which showed marked hyperexcitability, none of these monkeys showed any abnormality. Three of the monkeys were tested for circulating virus by the intracerebral inoculation of groups of mice. The titres of MEV circulating in the blood stream of these animals is presented in Table V. All of these rhesus monkeys developed antibody to MEV as a result of the inoculations.

C. aethops *monkeys.*

African grey monkeys (*Cercopithecus aethiops centralis* Neuman) are more susceptible than rhesus monkeys to inoculations of MEV.

Of 5 monkeys of this species inoculated with mouse-brain passage MEV, paralysis was present in all but one (594). This latter animal was inoculated intranasally with 1112 mouse intracerebral LD_{50} of virus. No circulating virus was demonstrated in 10 daily bleedings from this animal, and convalescent serum from it contained no neutralizing antibody to MEV.

The data on the inoculation of the other monkeys are presented in Table V. All these aethiops monkeys had elevation of temperature for 1 to 3 days between the 2nd and 7th days after inoculation. Aethiops 529, which was the only survivor in this group, showed a marked disinclination to use its left arm on the 6th day after inoculation. Complete paralysis of this arm was not present, and by the 10th day there was entire recovery. Fourteenth-day serum from this monkey showed the presence of neutralizing antibodies to MEV.

Aethiops 732 (Table V) was inoculated into the right thigh with MEV immediately after an intracerebral inoculation of 1 ml. of soluble starch into the right cerebral cortex. On the 6th day after inoculation the monkey was found to be dragging its right arm. Examination showed weakness of the right arm

TABLE V.—*Data of Inoculations and Titres of Virus Circulating in Blood Stream of Monkeys Inoculated with MEV, as Demonstrated by the Intracerebral Inoculation of Mice.*

Monkey. Species.	No.	Route of inoculation.	Inoculum intracerebral LD_{50}.	Source of inoculum-mouse passage.	Titre of virus in blood on day. 1	2	3	4	5	6	7	8
Rhesus	621	i.c.*	8,325,000	10th	0	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$	0	0	0
Rhesus	764	s.c.** (starch i.c.)	334	7th	0	$> 10^{-1}$	$10^{-3.46}$	$10^{-4.55}$	$10^{-3.00}$	10^{-1}	0	$< 10^{-1}$
Rhesus	826	i.v.†	334	7th	$> 10^{-3.40}$	$> 10^{-3.30}$	$10^{-4.70}$	$10^{-4.62}$	$> 10^{-4.30}$	$< 10^{-1}$	0	0
C. aethiops	529	i.c.	50,100	2nd	$< 10^{-1}$	$10^{-2.50}$	$10^{-3.50}$	$10^{-3.15}$	$10^{-1.52}$	$< 10^{-1}$	$< 10^{-1}$	0
C. aethiops	732	s.c. (starch i.c.)	1,112	38th	0	$< 10^{-1}$	$10^{-1.60}$	$10^{-2.00}$	$10^{-1.00}$	$10^{-1.32}$	$10^{-2.00}$	Died day 7
C. aethiops	627	i.v.	1,112	38th	$< 10^{-1}$	$> 10^{-2.25}$	$10^{-2.50}$	$10^{-2.45}$	$10^{-1.21}$	Sacrificed day 5.		
C. aethiops	626	s.c. & i.m.‡	70,000,000	18th	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$	$> 10^{-1}$	Died day 6.		

* i.c., intracerebral. ** s.c., subcutaneous. † i.v., intravenous. ‡ i.m., intramuscular.

and of the pectoral and shoulder muscles of the right side. On the 7th day it was found dead. At autopsy some generalized congestion of the brain was observed, probably largely post-mortem in nature. On the right side of the cerebral cortex a small congested area of about 2 mm. in diameter was noted, presumably the site of the starch trauma. Various portions of the brain and cord (as indicated in Table VI) were removed and weighed. Ten per cent suspensions were made of each portion with physiological saline as diluent, and groups of mice were inoculated with Seitz filtrates of each suspension.

The distribution of virus in the central nervous system of this monkey and in 2 other *C. aethiops* inoculated with MEV is compared in Table VI with that found in the original paralysed rhesus monkey (Dick, Smithburn and Haddow, 1948).

TABLE VI.—*Distribution of Virus in the Central Nervous System of* C. aethiops *Inoculated with MEV Compared with that of a Naturally Infected Paralysed Rhesus Monkey.*

Suspension of—	Mortality ratios of mice inoculated intracerebrally with Seitz-filtered suspensions of portions of central nervous system of—			
	C. aethiops.			Rhesus.
	732	627	626	UR 22
Cortex	3/6	—	1/6	0/6
Medulla and pons	0/6	—	2/6	0/6
Cerebellum	0/6	—	2/6	5/5
Midbrain	0/6	6/6	0/6	1/6
Cervical cord	1/6	—	4/6	3/6
Thoracic cord	2/6	—	6/6	—
Lumbo-sacral cord	6/6	—	6/6	6/6*

* Included portion of thoracic cord.
— Indicates no test made.

Aethiops 733 was caged with 732 before and after the inoculation of the latter, in order to determine whether the virus might be transmitted to monkeys by contact. Aethiops 733 showed no abnormality throughout an observation period of 23 days. It was bled daily for the first 10 days. No circulating virus was demonstrated, and the animal was non-immune at the conclusion of the experiment.

Aethiops 627 was inoculated intravenously. The results of titrations of the serum of each day's bleeding, presented in Table V, give ample evidence of the multiplication of this virus in aethiops monkeys, which is greater than that found in rhesus monkeys given a comparable inoculation. On the morning of the 5th day Aethiops 627 was found lying in its cage apparently unable to rise. Examination showed partial paralysis of the right lower limb. The right knee-jerk was less marked than the left, which may have been exaggerated. The abdominal reflexes were absent, and the abdominal wall was flaccid. The gluteal muscles and muscles of the back were weak. The monkey made no movement in response to painful stimuli of the lower limbs. This monkey was sacrificed on the 5th day, and the central nervous system was removed completely for histological material. A small portion of the midbrain was suspended in

saline, and MEV was demonstrated in this tissue by an intraperitoneal protection test in mice.

Aethiops 626 (Table V) had anorexia from the 3rd day after inoculation, and on the morning of the 5th day it exhibited partial paralysis of the left leg and was disinclined to use its left arm, although real paralysis in the latter could not be demonstrated with certainty. The animal was found dead on the morning of the 6th day. No gross abnormality was noted at autopsy. Groups of mice were inoculated with Seitz filtrates, and with the unfiltered supernate of suspensions of various portions of the central nervous system. Virus was demonstrated in greatest concentrations in the lumbosacral and thoracic portions of the spinal cord, and in lesser concentrations in other areas of the central nervous system (Table VI).

HISTOLOGICAL LESIONS INDUCED BY THE VIRUS.

In all the material that has been examined, the lesions induced by MEV have been restricted to the central nervous system (Fig. 1 to 10).

Monkeys.

The pathological material from monkeys has been limited, owing primarily to the small number of these animals that died or were sacrificed after inoculations of virus, and also to the fact that specimens in which post-mortem degenerative changes might have confused the picture have been avoided. In none of the monkeys was there any evidence of meningeal reaction or of abnormality of the ventricular system or its lining.

In the spinal cord of the paralysed rhesus UR 22 from which the virus was first isolated there was marked destruction of the ganglion cells, particularly in the anterior horns of the lumbar and cervical regions (Fig. 1 and 2). All stages of ganglion cell damage were seen. Many of the dying and dead ganglion cells were surrounded and invaded by macrophages (Fig. 2). In the medulla, pons,

EXPLANATION OF PLATES.

PLATE I.

FIG. 1.—Portion of lumbar cord of paralysed rhesus monkey showing cellular infiltration and destruction of ganglion cells. H. & E. × 200.

FIG. 2.—As Fig. 1 at higher magnification. H. & E. × 450.

FIG. 3.—Part of anterior horn of cervical portion of cord of a *C. aethiops* monkey showing various stages of ganglion cell damage in anterior horn. H. & E. × 450.

FIG. 4.—Part of pons of a paralysed *C. aethiops* monkey showing ganglion cell damage. H. & E. × 450.

PLATE II.

FIG. 5.—Area at base of brain of a paralysed guinea-pig, showing early nuclear changes in glial cells, but little cellular infiltration. H. & E. × 450.

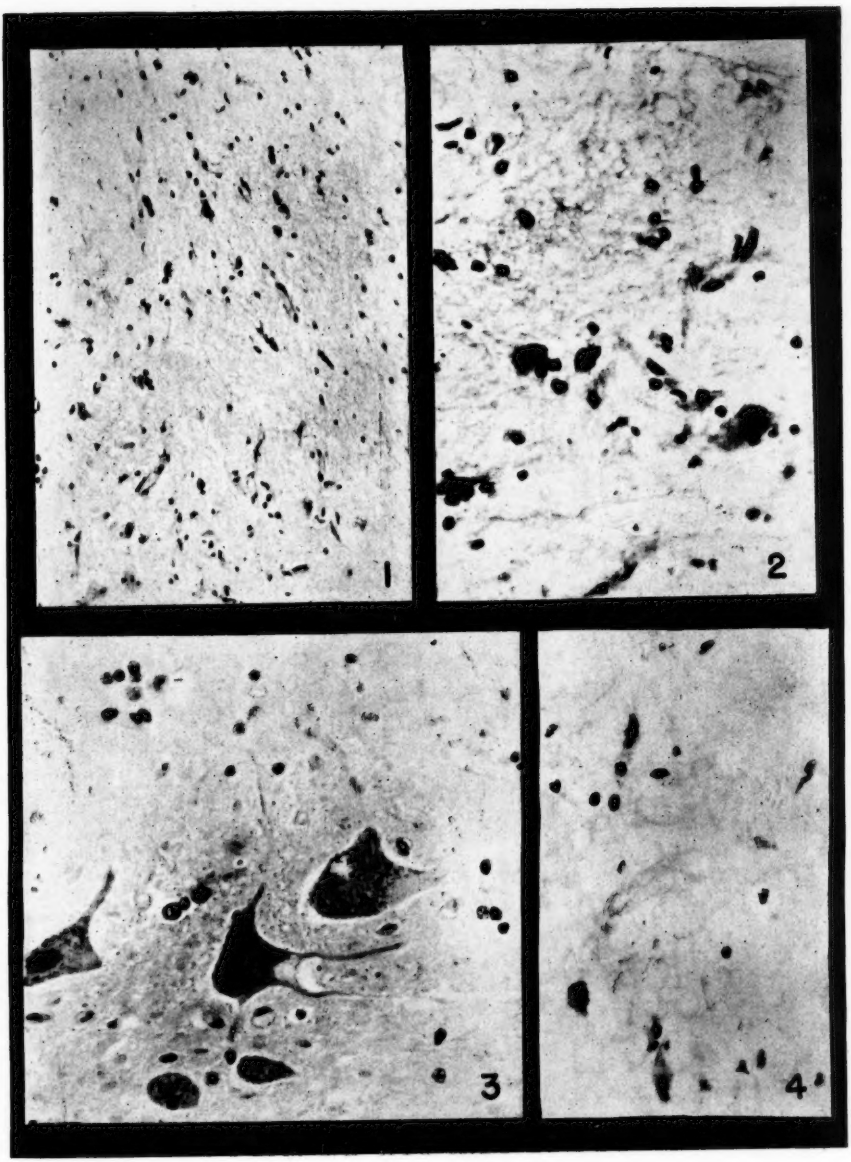
FIG. 6.—Area at base of brain of a paralysed mouse showing glial cell damage and cellular infiltration. H. & E. × 450.

FIG. 7.—Part of hippocampus of paralysed mouse showing area of necrosis of molecular layer. H. & E. × 30.

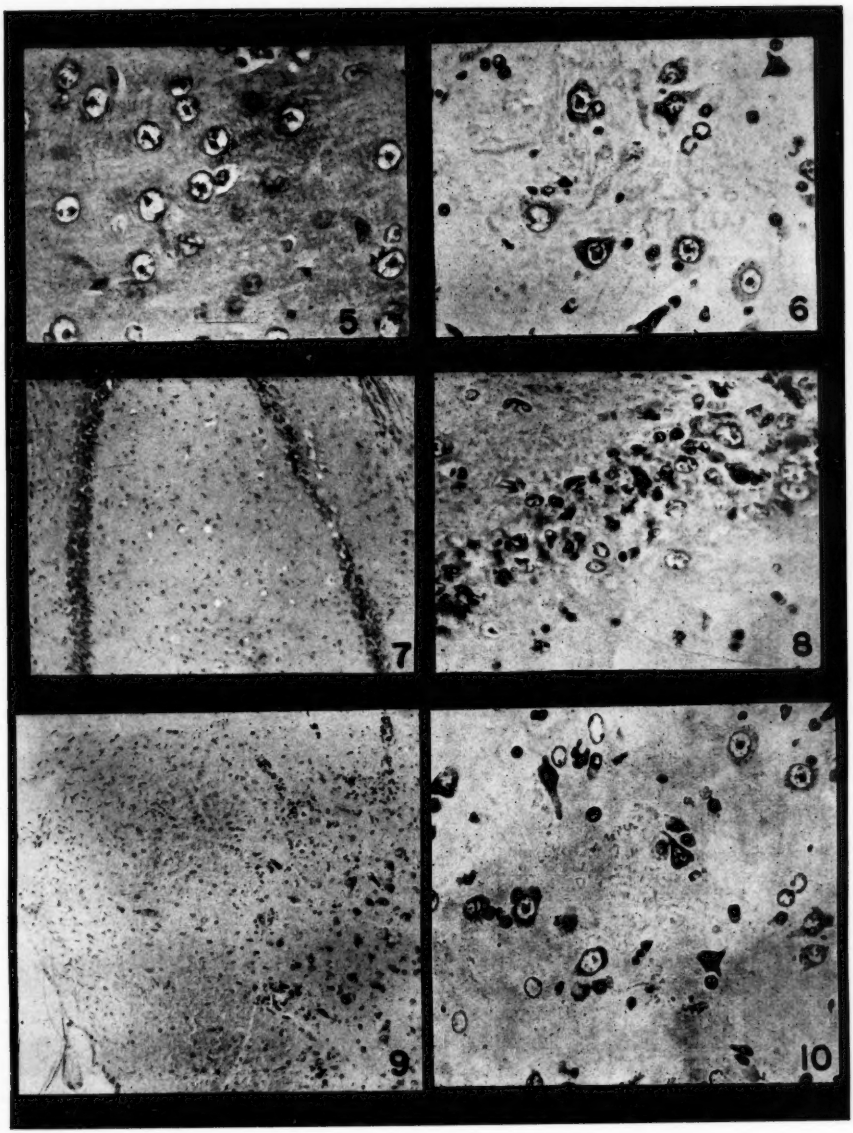
FIG. 8.—As Fig. 7 at higher magnification. H. & E. × 200.

FIG. 9.—Portion of cord of paralysed mouse showing cellular cuffing, infiltration and ganglion cell damage. H. & E. × 30.

FIG. 10.—As Fig. 9 with part of anterior horn at higher magnification showing various stages of ganglion cell damage. H. & E. × 450.



Dick.



Dick.

midbrain and cerebral cortex there was also evidence of nerve cell damage and generalized cellular infiltration of the grey matter, but cuffing of the vessels was less marked than in the cord.

Similar lesions were found, particularly in the upper cervical (Fig. 3) and lumbar region, in the cord of Aethiops 732 (Table VI). Nerve cell damage was most pronounced in the lumbar region. In this case there was little cuffing of the vessels, but all stages of degeneration of ganglion cells up to complete necrosis were present. Various portions of the brain of Aethiops 732 were examined. Apart from a certain amount of cellular infiltration, the cerebellum appeared normal and the Purkinjë cells showed no specific changes. In the cortex there was a fairly diffuse and widespread destruction of nerve cells, with neuronophagia, but very little evidence of perivascular cuffing. Marked ganglion cell damage was present in the pons (Fig. 4), but, as in the rest of the brain of this monkey, there was little evidence of perivascular infiltration.

In Aethiops 626 (Table V) there was evidence of specific ganglion cell damage in the cord, but there were minimal changes in the cerebrum, midbrain and pons. No cerebellar lesions were found.

Guinea-pigs.

The occurrence of pathological changes in the central nervous system of guinea-pigs was dependent on whether the infection was of an acute or of a more chronic nature. In Guinea-pig 337, which was sacrificed on the 2nd day after inoculation, the most severe damage was present in the hippocampus, in which there were large areas of complete necrosis in the molecular layer. There was little leucocytic reaction in these necrotic areas. The nerve cells throughout the brain and cord showed pathological changes (Fig. 5). The most characteristic changes were in the nuclei of the glial cells. In no place was there the complete margination of nuclear chromatin characteristic of certain viral diseases. The nuclei, however, showed a partial margination of chromatin, while the rest of the nucleoplasm was disintegrated into an eosinophilic nuclear dust. The nucleoli of the damaged nerve cells were eccentric, and could often be seen to have disintegrated into a dot of eosinophilic and a dot of basophilic material. Occasionally all that was left of the nucleus was the nuclear membrane to which the disintegrating basophilic and eosinophilic constituents of the nucleolus were adhering.

There was little cellular infiltration of the brain as compared with the cord, where the perivascular cuffing was marked. In this animal there were quite a number of polymorphs in the region of necrotic cells and around the blood vessels. These accumulations of polymorphs were not perivascular, but were arranged in foci at one side of the vessels. In another guinea-pig (338), which died on the 2nd day after inoculation, there was no necrosis seen in the hippocampus, but very severe glial cell damage was present in the midbrain and pons. Nerve cell damage was also present in the medulla and throughout the cord. In Guinea-pig 341, which was sacrificed on the 9th day after inoculation, there was a minimal amount of damage in the brain. In the cord of this animal, however, there were areas, particularly in the lumbar region, where destruction of the ganglion cells was complete. Throughout the cord there was marked infiltration with small round lymphocyte-like cells, and with mononuclear cells ingesting the necrotic material.

In none of the material from guinea-pigs was there evidence of any meningeal or ependymal reaction, and no nuclear or cytoplasmic inclusion bodies were seen.

Rabbits.

No pathological lesions were observed in material taken from inoculated rabbits.

Mice.

Material was obtainable from mice in the preparalytic stage and in the paralytic stage, in both early and late passage infections. In the brains of mice which were examined before the onset of objective signs of infection there was generally a certain amount of congestion, particularly of the pia. Damage to the nerve cells of all portions of the brain, with the exception of the Purkinjë cells, was in evidence (Fig. 6). The characteristic splitting of the nucleolus into its basophilic and eosinophilic constituents was present. The greatest damage in the brains of these mice was present in the hippocampus ; this was constantly most marked in the anterior part of the outer molecular layer. There was little evidence of cellular infiltration either in the brain or in the cord. In the cord, as in the brain, there was damage to the nerve cells.

In the central nervous system of mice which had become paralysed within 48 hours of inoculation there was marked necrosis of the hippocampus (Fig. 7 and 8). In some cases the outer molecular layer of the hippocampus contained a section of completely necrotic cells, and in most cases this necrotic section was quite clearly demarcated from cells in which minimal cellular changes were present. In all mouse brains examined these areas of necrosis have been confined to the anterior portion of the molecular layer of the hippocampus. In such necrotic areas very little cellular reaction was present, and there was only a small amount of infiltration of the adjacent white matter (Fig. 8). A variable amount of nerve cell damage was present in other portions of the brain, particularly in the base of the brain (Fig. 6). Throughout the cord there was evidence of nerve cell damage, and a moderate but variable amount of cellular infiltration of the grey matter (Fig. 9 and 10).

In mice which had been paralysed for several days the hippocampal lesions were often less marked. Areas of focal necrosis were present in various parts of the brain in which focal accumulation of glial nuclei were present. Compared with the mice in which there had been a rapid onset of paralysis, these mice showed a much greater diffuse cellular infiltration and more marked cuffing of the vessels, particularly in the spinal cord. Generally the cuffing was most evident in the lumbar portion of the cord. Owing to the presence of a certain amount of non-specific necrosis in the Purkinjë cells of nearly all normal mouse brains, probably due to the technical methods employed, the extent of damage produced in these cells by MEV was not readily estimated ; it was minimal, however, and was seen only in the brains of mice which had been paralysed for 5 or more days. In these mice the Purkinjë cells showed eosinophilic nuclear changes, loss of their processes and rounding up of the cell body.

The greatest amount of cellular infiltration was found in mice which had been paralysed for the longest periods. In such animals there was very marked cuffing of vessels both in the brain and spinal cord, and there were scattered

areas of focal necrosis. In some parts of the cord there were almost no nerve cells, and in some mice there was a diffuse infiltration of the white as well as the grey matter.

In the material examined no inclusion bodies were seen, and there was no evidence of endarteritis or vascular haemorrhages.

PHYSICAL PROPERTIES.

Filterability.

There was no appreciable decline in the titres of mouse-brain virus after passage through Berkefeld filter of grades V., N. or W. or through Seitz E. K. pads.

Size.

Estimations of the size of MEV were made by the method of Elford, Galloway and Perdrau (1935) at the Laboratory of the International Health Division of the Rockefeller Foundation during tenure of a Rockefeller Foundation fellowship. Several experiments were made, of which the following is a typical example: Brains were taken from 10 mice paralysed as a result of inoculation with 4th-mouse-brain passage MEV. A 10 per cent suspension of these brains was made in broth of pH 7·4. This suspension was centrifuged at 1500 r.p.m. for 15 minutes, and the supernate was passed through a Seitz E. K. pad. The Seitz filtrate was then put through a collodion membrane of 455 mμ. pore size. This latter filtrate was then passed through filters of 50, 45, 37, 30 and 22 mμ. size, at 8 lb. pressure. These filtrates were each tested by the intracerebral inoculation of 6 mice. No mice died as a result of inoculation with the 30 mμ. and 22 mμ. filtrates. There was 100 per cent mortality of mice inoculated with the other filtrates. From these experiments the particle size of MEV is estimated at 10 to 15 mμ., which is the estimated size of the virus of poliomyelitis (Theiler and Bauer, 1935; Elford, Galloway and Perdrau, 1935).

Potency of desiccated virus.

Several lots of mouse brain containing MEV were preserved by desiccation. The material to be desiccated was suspended in whole normal serum, the suspension was centrifuged and the supernate was distributed in Pyrex ampoules. This material was frozen and dried on a Flossorff-Mudd glass manifold, and the ampoules containing the dried virus were sealed with an acetylene flame while the vacuum pump was in operation. The dried material was tested periodically for potency. Although there was considerable loss of potency in the dried material after 10 days' storage, this material still contained virus after 18 months in the refrigerator.

Potency of virus preserved in glycerine.

Several lots of infected mouse brains were placed in a mixture of equal parts of glycerine and normal saline and were stored in a refrigerator. Titrations were made of 5 lines of virus thus preserved for periods of 9 to 10 months, and in only one case was the titre less than $10^{-5.50}$. The titre of virus in fresh mouse brain has seldom been under 10^{-6}. It may therefore be stated that up to 9

months there is relatively little loss in the virus content of mouse brains preserved in glycerine. The diminution in virus titre is much less in mouse brain thus preserved than in desiccated mouse brain.

Survival of virus in suspensions.

Experiments were done to discover the effect of sodium chloride on MEV. Brains of two paralysed mice of the 11th passage were suspended in distilled water to make a 10 per cent suspension. Serial decimal dilutions of this suspension were prepared with physiological saline and serum-saline. After the serial dilutions had been made, 0·5 ml. of saline was added to 0·25 ml. of the saline dilutions, and 0·5 ml. of normal serum was added to 0·25 ml. of the serum-saline dilutions. The mixtures were incubated for 1 hour at 37° C., and groups of mice were then inoculated intraperitoneally with 0·06 ml. of each mixture. The results of the experiment showed that the titre of virus in normal serum was $10^{-6.60}$ and the titre in the saline mixture $10^{-5.80}$, indicating that saline had only moderate effect on the potency of the virus.

Experiments were then carried out to compare the survival time of virus in saline and in serum-saline at refrigerator, incubator, and room temperature. In the first experiment brains of 40 paralysed mice of the 56th passage were weighed and then ground up with 7 volumes of distilled water. To 7 volumes of the Seitz-filtered virus suspension were added 1 volume of 8·5 per cent saline and 1 volume of normal monkey serum To another 7 volumes of the suspension 1 volume of 8·5 per cent saline and 1 volume of distilled water were added. Each batch was then distributed into ampoules in 51 lots. The ampoules were sealed, and one-third of each lot was kept in the incubator (37·5° C.), one-third at room temperature (21° to 25° C.) and the remainder in the refrigerator (2° to 10° C.).

Mice were inoculated at various intervals of time with serial dilutions of each suspension. No virus could be demonstrated on the 25th day in the suspensions in saline stored in the incubator (37·5° C.). No end-point was obtained with virus in serum-saline suspensions stored at this temperature, but the period of survival was more than 33 days (Table VII). The titre of virus suspended in

TABLE VII.—*Titres of MEV in Saline and Serum-Saline Suspensions Stored at Incubator Temperature.*

Time stored (days).	Titre of virus stored at incubator temperature (37·5° C.).	
	Saline suspension.	Serum-saline suspension.
0	$10^{-6.60}$	$10^{-6.60}$
7	$10^{-4.32}$	$10^{-5.60}$
13	$10^{-2.81}$	$10^{-3.42}$
25	$10^{-1.00}$	10^{-1} or >
33	—	10^{-1}

— No test made.

saline and stored at room temperature had dropped from $10^{-6.60}$ (on day 0) to $10^{-4.00}$ after 31 days. The titre of virus in serum-saline on the 31st day of storage at room temperature was $10^{-5.00}$. By the 102nd day of storage at this temperature virus was still present in the serum-saline suspension (titre $10^{-2.75}$). Un-

fortunately all the ampoules of virus suspended in saline were used by this time, and no comparison with saline suspension is available.

At refrigerator temperature there was more virus in the serum-saline suspension (titre $10^{-3.00}$) than in the saline suspension (titre $10^{-2.85}$) on the 90th day; on the 117th day of storage titres of $10^{-2.00}$ and $10^{-3.24}$ respectively were obtained.

In all experiments the loss of virus activity in the serum-free medium has been greatest during the first week of storage of the suspensions.

Thermal death point.

Suspensions of virus containing 10 per cent (by weight) of infective mouse-brains were made in 10 per cent monkey serum in physiological saline. The suspensions were centrifuged and passed through Seitz E. K. pads, and the filtrates were distributed in 0·5 ml. or in 1 ml. quantities into glass ampoules. After sealing, the ampoules were completely immersed in water baths maintained at the various temperatures required for the test. Ampoules were removed from each flask after the desired time intervals, and groups of mice were inoculated intracerebrally with 0·03 ml. of the contents of each ampoule.

It was found that when Mengo encephalomyelitis mouse-brain virus is suspended in 10 per cent serum-saline it withstands heating at 94° C. for 20 minutes, and that in this diluent it will even withstand heating at 98° C. for 5 minutes. All virus particles are destroyed when suspensions of mouse-brain virus are heated at 96° C. for 20 minutes, at 98° C. for 10 minutes or at 100° C. for 5 minutes. These observations of the marked thermal stability of suspensions of this virus in serum-saline were confirmed by many experiments.

All virus particles are destroyed, however, when a 10 per cent Seitz filtrate of mouse-brain virus in a serum-free diluent is heated at 60° C. for 30 minutes.

Using a similar technique to that described above, tests were made of the thermal stability of Mengo encephalomyelitis mouse-brain virus suspended in a diluent consisting of 0·1 per cent bovine albumin in saline buffered at pH 7·4 to 7·8. All mice inoculated with suspensions of MEV heated at 60° C., 70° C. and 80° C. for 30 minutes remained well over an observation period of 10 days. The groups of mice inoculated with suspensions of virus heated at 56° C. for 2 and 3 hours had mortality ratios of 6/6 and 5/6 respectively.

An experiment was done to make a direct comparison under identical conditions of the thermal stability of mouse-brain virus suspended in (*a*) 10 per cent serum-saline and (*b*) 0·2 per cent bovine albumin in saline. A 20 per cent suspension of infective mouse-brains was made in saline buffered at pH 7·4. After centrifuging at 2000 r.p.m. for 20 minutes the supernate was passed through a Seitz E. K. pad. This filtrate was divided equally. To one portion was added an equal quantity of 20 per cent monkey serum in saline, and to the other an equal quantity of 0·2 per cent bovine albumin in saline. The final concentrations were 10 per cent mouse-brain virus in (*a*) 10 per cent serum-saline and (*b*) 0·1 per cent bovine albumin in saline. These suspensions were heated as already described at 60° C., 70° C. and 80° C. for 30 minutes. The mortality ratios of mice inoculated with the contents of the ampoules are presented in Table VIII.

It may be seen from Table VIII that the thermostability of MEV is markedly greater when the virus is suspended in a diluent containing serum than when it is suspended in a serum-free medium. One hypothesis, from this experiment

TABLE VIII.—*Comparison of Thermostability of MEV when Seitz-filtered 10 per cent Mouse Brain Virus is Heated with 10 per cent Serum or with a Serum-free Diluent.*

Diluent employed.	Mortality ratio of mice inoculated with virus suspensions heated for 30 minutes at °C.		
	60	70	80
10 per cent monkey serum-saline	6/8	4/8	8/16
0·1 per cent bovine albumin-saline	0/8	0/6	0/16

and from the results of other experiments cited above, might be that when the virus is heated in a suspension containing 10 per cent serum, some of the virus particles become encased in coagulated serum proteins, which in some way insulate the virus and prevent its destruction by heat.

Susceptibility to chemicals.

A 10 per cent suspension of mouse-brain virus of the 95th passage was made and put through a Seitz filter. The filtrate was diluted with saline to 1 in 100 and distributed in test-tubes in 5 ml. quantities. Equal volumes of the chemicals to be tested were added to give the final concentrations shown in Table IX. The tests were made at room temperature (approximately 20° to 24° C.). The mixtures were inoculated intraperitoneally into groups of mice in 0·06 ml. quantities after the filtrates and the chemicals had been in contact for 12, 30 and 60 minutes, and 6, 24 and 72 hours.

It may be seen from Table IX that potassium permanganate in a concentration of 1 in 200 inactivated the virus within 12 minutes; at a concentration of 1 in 2000 there was some loss of virulence after 12 minutes of contact, but virus could still be demonstrated in this mixture up to at least 60 minutes. Active virus could be demonstrated in a 1 per cent hydrogen peroxide mixture after 24 hours of contact and in a 1 per cent phenol mixture after 6 hours. In a concentration of 0·5 to 1 per cent formalin the virus retained its activity

TABLE IX.—*Results of Tests of the Effects of Various Chemicals on the Viability of Mengo Encephalomyelitis Virus.*

Chemical.	Final percentage concentration.	Mortality ratio* in mice inoculated after contact of—					
		Minutes.			Hours.		
		12	30	60	6	24	72
Phenol	1	2/6	0/6	1/6	2/6	0/6	0/6
Formalin	1	6/6	5/6	5/6	0/6	0/6	—
	0·5	6/6	6/6	4/6	0/6	0/6	—
Alcohol	1	6/6	6/6	6/6	6/6	6/6	6/6
	5	6/6	6/6	6/6	6/6	6/6	6/6
Ether	50	6/6	6/6	6/6	6/6	6/6	5/5
Hydrogen peroxide†	1	6/6	6/6	6/6	6/6	4/6†	0/6
Potassium permanganate	0·5	0/6	0/6	0/6	0/6	0/6	—
	0·05	3/6	1/6	1/6	0/6	0/6	—

* See footnote, Table I.

† H_2O_2 titrated after 24 hours and found to be approximately 0·9 per cent.

beyond 60 minutes, and after contact with alcohol (1 to 5 per cent) and ether (50 per cent) for 72 hours it still showed marked activity.

DISCUSSION.

It has been shown that in monkeys and mice dying as a result of natural or experimental infection with the virus of Mengo encephalomyelitis, the largest amounts of virus are found in the spinal cord. In monkeys the lumbar portion of the cord appears to be the site of maximum concentration of the virus, irrespective of the route of inoculation. The number of monkeys from which material was taken for examination was not sufficient to warrant further statements concerning the distribution of the virus in these animals or its restriction to certain areas of the nervous system.

In monkeys infected with MEV either naturally or by inoculation the agent multiplies, as is evidenced by the rising titres of circulating virus, but it would appear that only in certain predisposed monkeys is the central nervous system selectively attacked. In mice inoculated intranasally, the titre of virus in the blood stream rises rapidly and reaches a peak in about 4 to 6 hours. No virus can be demonstrated in the brains of such mice until about the time the virus attains maximum concentration in the blood stream. This suggests that primary multiplication of virus may take place at a site outside the central nervous system prior to its invasion of that system. Once the central nervous system has been invaded, multiplication of virus seems to be limited to that system. At the time of maximum virus concentration in the brain and cord, virus can be demonstrated in the lungs, liver, spleen, kidney, heart and blood of mice, but the presence of virus in the visceral organs is probably to be accounted for by that present in the blood.

The virus is capable of inducing a true encephalomyelitis in man, monkeys, guinea-pigs, hamsters and mice.

Rhesus monkeys are less susceptible than *C. aethiops* monkeys to inoculations of MEV. Among inoculated rhesus monkeys only 2 out of 9 showed paralysis, and this was transient in nature. Both of these rhesus monkeys which became paralysed were inoculated with monkey-passage virus, and further studies on the susceptibility of rhesus monkeys to monkey-passage MEV are required. Of 23 unused rhesus monkeys from the outdoor runs of this laboratory, 8 were found to have become immune to MEV as a result of natural infections, but in none of them was any paralysis noted or recorded.

Aethiops monkeys are much more susceptible to inoculation of MEV than rhesus monkeys ; 4 or 5 animals of the former species became paralysed after inoculation. Of 6 aethiops monkeys bought locally and caged in the Institute compound 1 had antibody to the virus, but none of these monkeys has been found paralysed in nature. Studies have not as yet been made to discover whether in non-paralytic infections with this virus there is histological evidence of nerve cell destruction.

In infection with MEV significant lesions have not been observed in any tissue except that of the nervous system, which appears to be selectively invaded and damaged, and the available information indicates that MEV is highly neurotropic. There is considerable microscopic evidence that this virus primarily damages the nerve cells, and that infiltration and cuffing of vessels are secondary

in nature. Sufficiently extensive studies on the distribution of the lesions in the central nervous system of monkeys infected with MEV have not been made. In mice the cord lesions caused by MEV bear resemblances to the lesions produced by Theiler's encephalomyelitis virus of mice and by the Jungeblut and Sanders strains of rodent-adapted human poliomyelitis virus (Theiler, 1937 ; Jungeblut and Sanders, 1940 ; Jungeblut, Feiner and Sanders, 1942 ; Jungeblut, Sanders and Feiner, 1942). Experiments have been planned to determine whether MEV is related antigenically to the human strain of poliomyelitis virus and to the Lansing (Armstrong, 1939*a*, 1939*b*) and the Jungeblut and Sanders rodent-adapted strains of poliomyelitis virus.

Probably the most interesting physical properties of this virus are its marked stability under various conditions and its remarkable resistance to inactivation by heat and various chemicals.

SUMMARY.

Strains of Mengo encephalomyelitis virus (MEV) isolated from a human patient, 2 rhesus monkeys, a mongoose and a batch of *Taeniorhynchus fusco-pennatus* mosquitoes caused 100 per cent mortality in inoculated mice by the 2nd intracerebral passage. Virus isolated from a mixed group of mosquitoes of the *Taeniorhynchus* genus killed all mice in one line of transfer by the 3rd passage, and all in other lines by the 4th and 6th passages. From the earliest passages the virus was found to be pathogenic for mice when inoculated intracerebrally, intraperitoneally, intranasally or subcutaneously. In the early passages in adult mice, the first clinical evidence of illness was as a rule paralysis of one or more limbs. In the later passages death usually occurred within 24 to 48 hours after inoculation without any preceding evidence of paralysis or sickness.

Guinea-pigs were found to be susceptible to inoculation of the virus by intracerebral, subcutaneous, intradermal and intraperitoneal routes. Juvenile guinea-pigs were more susceptible than older ones to this virus. Paralysis was observed in only 3 of the 22 inoculated animals. Circulating virus was demonstrated in all animals tested except one. Antibody was demonstrated in the convalescent sera of all animals that survived.

Rabbits appeared to be relatively insusceptible to the intracerebral inoculation of MEV of mouse-brain passage. While virus was shown to be present in the brains of some inoculated animals, there was no evidence of multiplication of virus, and passage of rabbit brain to rabbits did not produce encephalitis in the subinoculated animal.

In rhesus monkeys inoculated with, or naturally infected with, Mengo encephalomyelitis virus, paralysis appeared to be a rare manifestation of the activity of the virus. Only 2 of 9 rhesus monkeys inoculated showed any indication of paralysis, and no paralysis was noted or recorded in 8 (35 per cent) monkeys of this species from the outdoor run of the laboratory which were found by sera tests to be immune to the virus. Aethiops monkeys are much more susceptible to inoculation with the virus than are rhesus monkeys. Four out of 5 monkeys of this species became paralysed after inoculation with MEV. Naturally occurring immunity has also been demonstrated in this species.

The pathological lesions induced by the virus in monkeys, guinea-pigs and mice are described.

MEV passes through all grades of Berkefeld filters with no appreciable loss in titre. The size of the virus is estimated at approximately 10 mμ., which is of the same order as that of the virus of poliomyelitis. The relationship (if any) of MEV to the Lansing and Jungeblut and Sanders strains of rodent-adapted human poliomyelitis virus has not yet been determined.

The remarkable stability of this virus in suspensions, the high thermal stability in diluents containing serum and the effects of certain chemicals on its viability are described.

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THE ANTIGENIC FUNCTION OF SIMPLE CHEMICAL COMPOUNDS: CORRELATION OF ANTIGENICITY WITH CHEMICAL REACTIVITY.

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IT is known that the parenteral inection into rabbits of a number of low-molecular-weight compounds which can react with proteins stimulates antibody production (Gell, Harington and Pitt Rivers, 1946). In that study it was pointed out that "it is reasonable to anticipate that those compounds which are hydrolysed with extreme ease will be relatively ineffective in forming antigens *in vivo*; the more stable ones will not only be more likely to react with amino-groups before hydrolysis occurs, but they may also in part survive unchanged, as already suggested, until they are absorbed by phagocytes, in which they may find an environment more suitable for conjugation." The present study describes an attempt to account, in terms of their chemical reactivity, for the marked variation in antigenicity between some of these compounds. For this purpose three compounds were selected, namely, benzyl chloroformate, 2-phenyl-4-ethoxymethylene-oxazolone and *p*-nitrobenzoyl chloride. The two first were used in our previous investigation, in which their powers of provoking antibody formation were shown to be strikingly different. The third was selected owing to its very much greater rate both of hydrolysis and of conjugation.

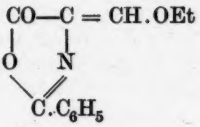
The reactivity of the compounds was measured in the presence and in the absence of alanine; this amino-acid was chosen both because of its convenience of estimation, and because the pK of its amino-group is similar to that of the free amino-groups in protein. In this way the rate of hydrolysis of the compounds under examination could be compared with the rate of coupling on to $-NH_2$. *A priori*, one would expect that both the absolute rate of the reaction with $-NH_2$ groups and the ratio of this rate to the rate of hydrolysis (i.e. the affinity for amino-groups) would have an effect on antigenicity. A third factor likely to affect antigenicity, namely, the nature of the substituent group itself, was not under control in the substances at our disposal.

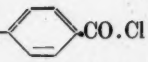
The antigenicity was measured by the injection of the simple substance, dissolved in acetone, into rabbits. Both the intravenous and the intraperitoneal routes were used. An essential difference between these routes is that in the blood stream the quantity of substance injected would immediately come into the presence of a large excess of protein; in the peritoneum the amount of protein would increase during some hours while an inflammatory exudate was collecting.

MEASUREMENT OF ANTIGENICITY.

Preparation of Antisera.

The substances used as pro-antigens were :

A. 2-Phenyl-4-ethoxymethyleneoxazolone, 

B. *p*-Nitrobenzoyl chloride, 

C. Benzyl chloroformate, $C_7H_7O.CO.Cl$ (Carbobenzyloxy-chloride).

(hereafter referred to as A, B and C).

The substances were dissolved in acetone just before injection at a concentration of 0·2 M; a twenty-fold dilution (to 0·01 M) in acetone was made from this. Eight rabbits were used for each substance. They were divided into pairs, one pair receiving the 0·2 M solution intravenously (into an ear-vein), one pair the same dilution intraperitoneally ; the other two pairs were similarly treated with the 0·01 M dilution. Thus for the three substances 24 rabbits were used. All the animals were " Sandy lops " from the Medical Research Council stock.

A quantity of 0·1 ml. per injection was used and the animals were given two 3-week courses of 6 injections, with an interval of four weeks between the courses. About half an hour before the first injection of the second course, each animal was given 20 mg. of Benadryl subcutaneously, as a precaution against anaphylactic shock. There were no losses from this or any other cause during the experiment.

A very small sample of blood was taken four days after the end of the first course, and four days after the last injection the animals were bled from the ear, 40–80 ml. being obtained from each animal. The serum was separated by spinning, sodium azide to a concentration of 0·1 per cent was added as a preservative, and all the samples were kept in the cold-room at 4° C. until tested. The sera were aged for 12 weeks before testing, but were not inactivated.

Intravenous injection of acetone solutions, even in a volume of only 0·1 ml., inevitably damages the veins of the ear, and it is often impossible to prevent thrombosis of the vein used. In lop-eared animals, however, there are many good veins available, and if the vein is carefully massaged immediately after the injection thrombosis may be entirely avoided. In any event the circulation is fully re-established in time. Acetone is no doubt not the best vehicle, but our experience suggests that it is desirable to have the substance properly in solution when injected, to get a satisfactory antibody response. No difficulty was experienced with the intraperitoneal injections.

Preparation of Antigens for Precipitin Tests.

The methods used in the preparation of antigens 130 (carbobenzyloxygelatin) and 135 (*p*-nitrobenzoylgelatin) were essentially similar to those used in our previous work, and need not be described.

A slightly different method was used in the preparation of antigen 138 (2-phenyl-4-methyleneoxazolonogelatin).

Antigen 138.—600 mg. of gelatin were dissolved in 30 ml. of distilled water; 300 mg. of phenylethoxymethyleneoxazolone were dissolved in 10 ml. of a 10:3 chloroform-amyl alcohol mixture. The two solutions were shaken together thoroughly to emulsify, a few drops of phenolphthalein solution were added, and the pH adjusted with fresh 0·5 N KOH to give a weak pink colour. The mixture was shaken by hand at intervals, and more alkali was added from time to time as the pH declined. The reaction was allowed to continue for 4 hrs., and the mixture was then precipitated with alcohol and a little NaCl; the precipitate was redissolved in saline, reprecipitated with alcohol, washed several times with acetone and dried over P_2O_5 *in vacuo*.

Analysis of antigens for hapten content.

Antigens No. 130 and No. 138 were analysed for hapten content by the methods described in our previous report. Antigen No. 135 was analysed after hydrolysis, making use of the Kapeller-Adler reaction, by the same method as that used for phenylalanine (Michel, 1944).

Analysis of p-*nitrobenzoylgelatin.*—300 mg. of Ag. 135 were boiled under a reflux condenser for 9 hours with 10 ml. of 5N HCl. The hydrolysate, concentrated to dryness *in vacuo*, was extracted with 125 ml. ether and then with 25 ml. dry acetone. The extracts were evaporated to dryness; the residue was dissolved in a few ml. of very dilute KOH and transferred quantitatively to a 20 ml. graduated cylinder. The colour of an aliquot of this solution was read in a Pulfrich photometer, using a 560 mμ. filter and a 1 cm. cell. The concentration of *p*-nitrobenzoic acid was then read from a calibration curve, made by the same method with the pure substance. The results of the analysis are given in Table I.

TABLE I.—*Composition of Antigens.*

Antigen No.	Determinant group.	Molecular weight.	Per cent hapten content (anhydrous).	Groups per molecule (gelatin M.W. = 18,000).
130	Carbobenzyloxy-	135	2·5	3·4
135	2-Phenyl-4-methylene-oxazolono-	150	5·4	6·8
138	p-Nitrobenzoyl-	173	5·8	6·4

Although the hapten content of Antigen 130 is not high, previous work with it has shown that it is as powerful antigenically as, or slightly better than, one containing about 10 groups per molecule.

For use the antigens were dissolved at a concentration of 5 mg. per ml. on the basis of anhydrous gelatin, in saline containing 0·1 per cent sodium azide, 0·05 per cent sodium acetate and a trace of phenol red. The pH was adjusted to 7·2–7·4 before use.

Method of Estimation of Antibody.

The appropriate quantity of serum (usually 0·5 or 1 ml.) was put into a 2½-in. by 5/8-in. round-ended centrifuge tube and the antigen added, also usually in a volume of 1 ml. or less. A control tube containing serum only was included in each batch. The reaction was allowed to proceed at room temperature until a macroscopic precipitate had appeared; the total volume in the tube was then

made up to about 6 ml. with saline, and the tubes were capped with tinfoil and put at 4° C. for 5–7 days. They were then spun, the precipitates washed three times with 6 ml. volumes of saline, and the protein content of the precipitate estimated by means of Folin-Ciocalteau (phenol) reagent. For this purpose 2 ml. of 0·5 N NaOH were added to each tube, and the tubes were held at 37° C. for 20 to 30 min. to dissolve the precipitate. The volume was made up to 8 ml. with distilled water, and 0·4 ml. of the reagent was added rapidly from an automatic syringe. Immediately after this the contents of the tube were mixed by inversion on to a slip of cellophane. (By this method no "clouding" occurred, even after several hours.) The colour was allowed to develop for 30 min., and then estimated in an "Eel" colorimeter against the control tube as a blank (though this never gave more than a very slightly higher reading than a reagent blank). The concentration of protein was adjusted as far as possible to give a reading of between 20 and 80 on the colorimeter scale (about 0·25 to 1·00 mg.).

The micro-ammeter reading was converted to antibody concentration from a calibration made with known amounts of rabbit γ-globulin, using the above colorimetric technique. The calibration curve was linear from 0 to 2 mg., and multiplication of the micro-ammeter reading by an empirical factor of 0·141 gave the amount of globulin in mg. A correction was applied to the readings in Series A, since the antigen in this case gave an appreciable colour with the Folin-Ciocalteau reagent. The method of applying this correction is described in our previous report; it amounted to a reduction of rather less than 10 per cent at the optimum. The other two antigens gave no colour with the reagent.

Results.

The results of the estimations are given in Table II, and shown graphically in Fig. 1–3. For ease of calculation the antigens have been reckoned as 1 per cent, i.e. the actual dilutions have been multiplied by 1/2.

The preliminary samples of sera, taken after only one course, were estimated rather roughly, and have not been included in the table. In this case the pairs of sera were combined. The antibody responses to B and C were about equal, while that to A was superior, particularly in the intraperitoneal series. The second course did not improve the antibody content of some of these sera.

Although no definite deterioration seems to have occurred during the preliminary ageing of the sera, some of those of Series A gave results almost 20 per cent lower on re-testing after about 14 days, and were evidently "going off." A mean value has been adopted for these; there would in any case be no difference in the order of merit of the sera whichever series of values were taken. Series B and C gave good checks at this time.

The values in heavy type in Table II are those for the first tube where antigen was not detectable in the supernatant. The last column gives the value of n for the molar ratio A_nG_2 for this tube, i.e. if the antigen and antibody were present in equimolar amounts the value in this column would be 2. These values will be discussed in a subsequent section.

MEASUREMENT OF CHEMICAL REACTIVITY.

The reactions were carried out at 37° C. and at three different pH values, the pH being kept stable by the use of buffers. Alanine was used as a model substance to indicate the reactivity with amino-groups; this amino-acid has the advantage

TABLE II.—*Mg. of Antibody Precipitated from 1 ml. Serum with Varying Amounts of Antigen (as γ-Globulin).*

Serum No.	Pro-antigen.	Route.	Concentration: 0·1 c.c. per dose.	Dilution of antigen as 1 per cent: 1 ml. volume.											For A_nG_2 n =
				2/1.	1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.	1/256.	1/512.	
				Mg. of antigen per ml. serum.											
				20.	10.	5.	2·5.	1·25.	0·62.	0·31.	0·16.	0·08.	0·04.	0·02.	
51	A	I-Per.	0·2 M	—	0·8	—	2·8	—	2·7	**2·6**	—	1·4	—	—	2·5
53	„	„	„	0·64	5·0	—	4·6	—	**4·2**	3·6	—	2·0	—	—	2·0
58	B	„	„	—	—	—	—	0·29	0·58	0·66	**0·62**	0·52	0·37	—	1·2
59	„	„	„	—	—	—	—	0·11	0·12	0·25	0·31	**0·35**	0·32	—	1·3
52	C	„	„	—	—	—	—	0·48	0·72	0·68	**0·71**	0·55	—	—	1·4
62	„	„	„	—	—	—	—	0·0	0·41	0·48	**0·43**	0·44	—	—	0·8
54	A	I-Ven.	„	—	—	—	0·65	0·94	0·94	0·91	0·83	**0·78**	—	—	3·0
56	„	„	„	—	—	—	0·83	10·4	1·10	0·99	0·93	0·87	—	—	3·3
63	B	„	„	—	—	—	—	0·75	1·13	1·24	**1·20**	0·95	0·52	—	2·3
65	„	„	„	—	—	—	—	0·36	0·40	0·48	0·49	**0·46**	0·30	—	1·8
60	C	„	„	—	—	—	—	0·41	0·62	0·64	0·59	**0·43**	—	—	1·7
64	„	„	„	—	—	—	—	1·10	1·22	1·10	**0·93**	0·61	—	—	1·8
55	A	I-Per.	0·01 M	—	—	—	—	—	—	—	0·16	0·15	0·14	**0·15**	2·3
61	„	„	„	—	—	—	—	—	—	—	0·05	0·06	0·05	**0·07**	1·1
66	B	„	„	—	—	—	—	—	—	—	0·08	0·11	0·11	**0·11**	1·8
67	„	„	„	—	—	—	—	—	—	—	0·00	0·03	0·05	**0·04**	0·6
70	C	„	„	—	—	—	—	—	—	—	0·09	0·14	0·13	**0·11**	1·8
72	„	„	„	—	—	—	—	—	—	—	—	0·05	0·07	**0·06**	0·9
69	A	I-Ven.	„	—	—	0·0	0·0	0·49	0·56	0·62	0·63	**0·75**	—	—	2·9
71	„	„	„	—	—	0·0	0·72	0·96	1·03	1·21	**1·33**	1·34	—	—	2·5
68	B	„	„	—	—	—	—	—	0·14	0·16	0·17	0·19	**0·19**	0·16	1·4
74	„	„	„	—	—	—	—	—	0·11	0·23	0·34	0·40	**0·34**	0·17	2·6
73	C	„	„	—	—	—	—	—	—	—	0·14	0·15	0·14	**0·11**	1·8
75	„	„	„	—	—	—	—	—	—	—	0·16	0·17	0·14	**0·11**	1·8

Each number indicates a serum from a single animal.
Pro-antigens: A = Phenylethoxymethyleneoxazolone; B = *p*-nitrobenzoyl chloride; C = benzyl chloroformate.
Abbreviations: I-Per. = intraperitoneal; I-Ven. = intravenous.
For the figures in the final column see Discussion below.
The values in heavy type indicate the first tube where antigen was not detectable in the supernatant ("Equivalence point"). The ratios in the last column are calculated for this tube.

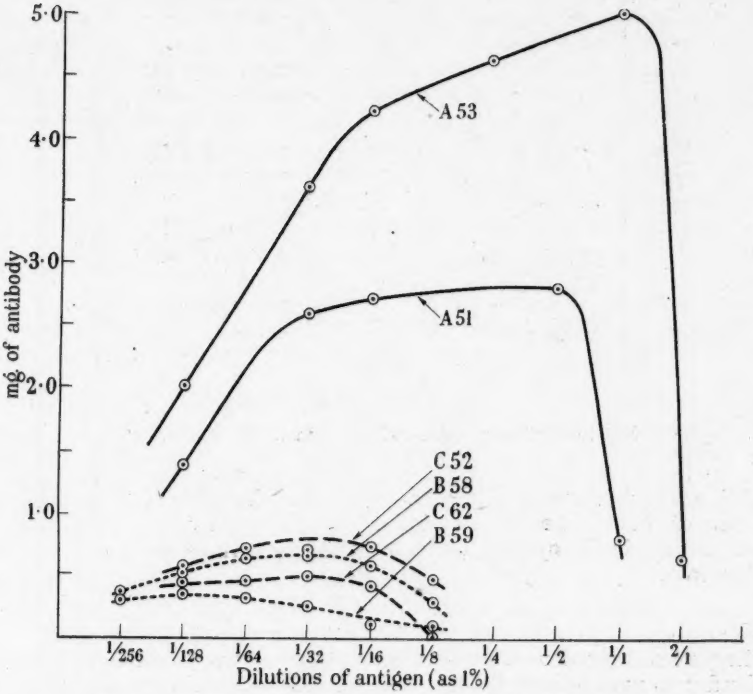


FIG. 1.—Antibody content of sera; large dose, intraperitoneal.

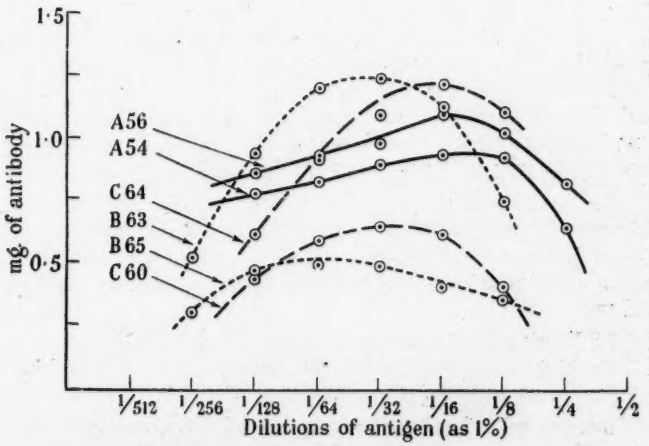


FIG. 2.—Antibody content of sera; large dose, intravenous.

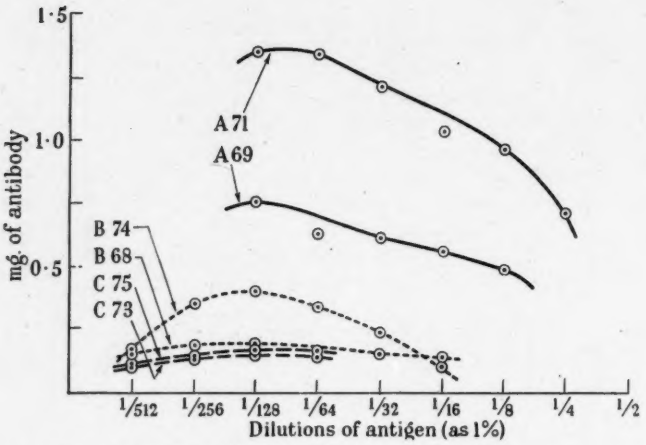


FIG. 3.—Antibody content of sera ; small dose, intravenous.

that it is easily determined in small amounts, and that the pK of its amino-group is similar to that of the free ε-amino groups of protein. The concentration of reacting groups in the system used was of the same order as that in the animal blood stream.

Method.

The general method used for all three compounds was to shake the compound, dissolved in a water-immiscible solvent, with a buffered aqueous equimolecular solution of alanine, using aqueous buffer without alanine as a blank each time. After a given length of time the total amount which had reacted was measured by determining either unchanged material in the organic solvent, in the case of the oxazolone, or chloride ions in the aqueous phase in the case of the other two compounds ; an estimation of unreacted alanine then gave, by difference, the proportion of the substance which had combined with the amino-acid. The point for zero time was obtained by mixing the reagents in an ice-bath, shaking, filtering and doing the estimations as quickly as possible. It will be observed that for benzyl chloroformate there is always a small positive value at this point, showing that the sample used was already slightly decomposed.

A. *2-Phenyl-4-ethoxymethyleneoxazolone.*

In a 100 ml. Erlenmeyer flask were placed 11 ml. of buffer solution, 0·5 ml. of a 6 per cent aqueous alanine solution (0·33 millimole) and 4·5 ml. toluene. The buffers used were :

pH 6·7 : Equimolecular amounts of mono- and di-potassium phosphate.
pH 7·4 : 20·4 g. KH_2PO_4 in 100 ml. water + 125 ml. 1 N NaOH.
pH 8·5 : Solution of $NaHCO_3$ saturated at 15° C.

In another flask 0·5 ml. of water was substituted for the alanine solution. The mixtures were shaken, warmed to 37° C., and 0·33 millimole of the oxazolone,

dissolved in 0·5 ml. toluene, was added rapidly to each. The flasks were then put on a "Microid" shaker, which was run at constant speed; this was taken as the beginning of the timed period. After a predetermined time the shaker was stopped and the solution rapidly filtered through a folded paper damped with 2 ml. of water; the first part of the filtrate was returned to the filter-paper. The analysis was then done as quickly as possible. An aliquot of the residual toluene was taken and the nitrogen estimated by the Kjeldahl method; this gave the amount of undecomposed oxazolone since, in the presence of buffers (both at pH 7·4 and at pH 8·5), the phenylethoxymethyleneoxazolone alone remains entirely in the toluene, the hydrolysis product (phenylhydroxymethyleneoxazolone) and the alanine derivative passing into the aqueous phase.

The amount of free alanine in the filtrate was determined by a modification of the method of Alexander and Seligman (1945) based on determination of acetaldehyde after reaction of the amino-acid with ninhydrin. None of the alanine derivatives of the substances under test are decomposed under these conditions.

Determination of free alanine.—At least 5 ml. of the solution to be analysed, adjusted to pH 5·5 and diluted if necessary to contain 300–800 μg. of the amino-acid, were put in a flask; to this were added 1 ml. of a freshly prepared 1 per cent aqueous solution of ninhydrin, 2 ml. of 2 M phosphate buffer pH 5·5, and a glass bead. The flask was attached to a condenser, to the end of which a tube was fitted containing 8 ml. of an ice-cooled 1 per cent solution of sodium bisulphite at pH 6·7. A current of air was led through the apparatus, the entering air being bubbled through sulphuric acid. The mixture was boiled for 75 min., during which time the liberated acetaldehyde was fixed by the bisulphite. The bisulphite solution was transferred quantitatively to a flask and the excess bisulphite neutralized exactly with 0·005 N and 0·1 N iodine in the presence of a little starch. 5 ml. of a saturated solution of $NaHCO_3$ were added to decompose the bisulphite compound and the bisulphite liberated was titrated with 0·005N iodine. One ml. of 0·005 N iodine is equivalent to 0·2225 mg. alanine.

B. *p-Nitrobenzoyl chloride.*

The method described above was used for this compound, except that anisole was substituted for toluene as a solvent. Total hydrolysis was measured by a Charpentier-Volhard titration of chloride ions.

Determination of chloride.—An aliquot of less than 5 ml. was taken, since an excess of salt affects the accuracy. Five ml. of HNO_3 diluted one in five, 1 ml. 0·1 N $AgNO_3$ and 2·5 ml. of a saturated solution of ammoniacal iron alum were added. The excess $AgNO_3$ was titrated with a freshly prepared solution of 0·005 N ammonium thiocyanate.

Unreacted alanine was determined as described above.

C. *Benzyl chloroformate.*

The method used with this compound was as described under A, except that double volumes were used, the concentrations being the same. The total hydrolysis was determined by chloride estimation, as before.

Results of chemical tests.

These are summarized in Table III. The actual values of the estimations are shown in column i for the blank, and columns iii and iv for the reactions in the presence of alanine. The other columns are derived from these experimental data, and the results are discussed in the next section.

TABLE III.—*Chemical Reactivities.*

Substance.	pH of buffer.	Duration of shaking (hr.).	Blank.			Test.					
			Mg. N in toluene (for A) or Mg. HCl liberated (for B and C). i.	Per cent decomposition (total). ii.	Mg. N in toluene (for A) or mg. HCl liberated (for B and C). iii.	Mg. alanine uncombined. iv.	Per cent decomposition (total). v.	Per cent combined alanine. vi.	Hydrolysis in presence of alanine (v—vi). vii.	Ratio of conjugation to hydrolysis (vi/vii). viii.
A	7·4	0	4·52	0	4·52	30·0	0	0	..	..
		4	4·08	9·7	3·62	25·0	19·9	16·7	3·2	5·0
		7	3·93	13·2	3·06	23·2	32·3	22·6	9·7	2·3
		15	3·50	22·6	2·08	17·3	54·0	42·3	11·7	3·6
	8·5	0	4·48	0	4·48	30·0	0	0	..	..
		3	4·10	8·45	1·27	4·7	7·15	68·4	3·1	22·0
B	7·4	0	..	0	0	30·0	0	0	..	..
		½	..	4·29	35·2	6·72	18·0	55·1	40·0	15·1	2·7
		1¼	..	8·83	72·4	11·0	13·4	90·2	55·3	34·9	1·6
		1½	..	10·4	85·2	11·8	11·3	96·6	62·3	34·3	1·8
		4	..	12·0	97·6	12·3	11·0	100·0	63·3	36·7	1·7
C	6·7	0	..	0·5	0	0·5	60·0	0	0	..	..
		3	..	1·97	6·18	2·27	56·7	7·44	5·5	1·9	2·8
		7	..	3·75	13·7	48·8	54·3	9·5	8·7	11·7	1·1
		15	..	7·71	30·3	8·78	52·1	24·8	13·1	11·7	1·1
	7·4	0	..	0·5	0	0·5	60·0	0	0	..	..
		1	..	1·26	3·19	2·18	56·7	7·05	5·5	1·5	3·5
		2	..	2·06	6·55	3·83	55·0	14·0	8·3	5·7	1·5
		4	..	2·91	10·3	6·36	52·1	24·6	13·2	11·4	1·2
		7	..	4·24	15·7	9·58	47·3	38·2	21·2	17·0	1·2
		15	..	8·27	32·6	15·4	38·0	62·6	36·6	26·0	1·4
	8·5	0	..	0·5	0	0·5	60·0	0	0	..	..
		2	..	3·39	12·1	9·4	39·6	37·4	34·0	3·4	10·0
		4	..	7·41	29·1	15·6	28·6	63·3	52·3	11·0	4·7

DISCUSSION.

Taking first the data on the chemical reactivities of the substances under test, Table III and Fig. 4 are designed to bring out certain points. First, it may be noted that all these substances have a marked "preference" for amino-groups; this is seen on comparing columns ii and vii of Table III, which show the per cent hydrolysis in the absence and in the presence of alanine. When there are amino-groups available, the rate of the reaction with water is much reduced, although, of course, the total decomposition (col. v) is increased. Column viii is designed to define the "efficiency" of the substance as a conjugating agent;

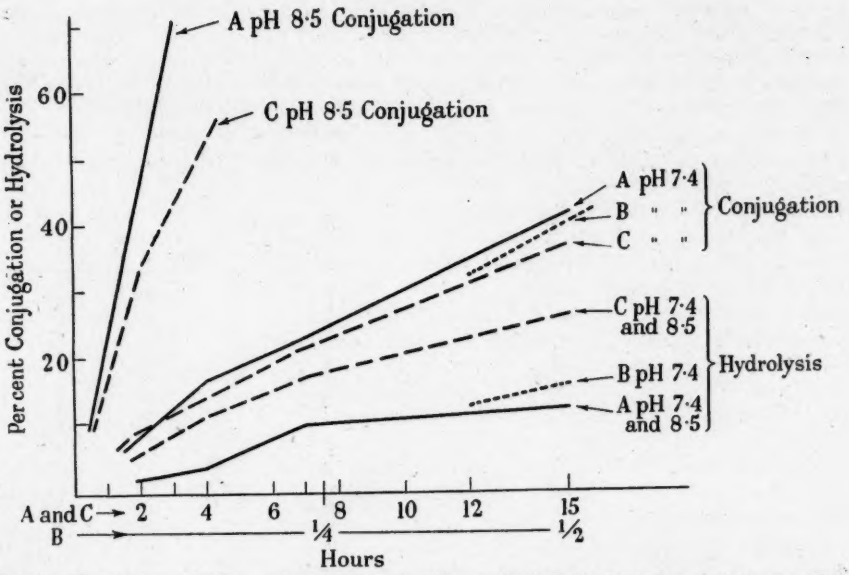


FIG. 4.—Chemical reactivity; percentage conjugation or hydrolysis plotted against time. (Note different time-scale for substance B.)

if the time-factor is ignored, the larger the proportion of the substance combining with $-NH_2$ (and so with protein), the more effective it is likely to be in "loading" body-proteins with hapten groups to make them antigenic. The higher ratio in the initial stages may also be noted, this being presumably due, in part at least, to the gradual reduction in the concentration of free $-NH_2$ as conjugation proceeds.

The relative efficiency of the substances is illustrated graphically in Fig. 4, where the values in cols. vi and vii of Table III are plotted against time. Here the wider the "angle" between the curves for hydrolysis and for conjugation, the more efficient the agent. The values for hydrolysis of A and C at pH 7·4 and 8·5 are so close (at least where data are available for pH 8·5 in the early stages) that they have been plotted as coincident. The values for B are on a time-scale 30 times that for the others, in order to bring them into the same range. The rapidity of decomposition of substance B at pH 8·5 made its kinetics at this pH impossilbe to measure by our methods; if, by analogy with the values at pH 7·4, its 50 per cent conjugation point is intermediate between those for A and C, this amount of conjugation would occur in about 4 minutes. At any rate it is clear that, ignoring for the moment the time factor, the efficiency of conjugation of the substances is in the order A>B>C.

Is there any reflection of this in the biological tests? The antibody contents of the various sera are plotted in Fig. 1–3 (the values for the small-dose, intraperitoneal group are not worth illustrating); in Fig. 1 (large dose, intraperitoneal), and to a lesser extent in Fig. 3 (small dose, intravenous), the series A sera seem

to have a decided advantage. Otherwise there is no definite advantage to any substance, and B and C are throughout apparently about equal. This suggests that the rapidity of reaction of B is a disadvantage, outweighing its favourable conjugation/hydrolysis ratio, which ought to bring it very near to A in efficiency. With this proviso the biological tests may be said to march with the chemical.

Now one can understand why, by the intraperitoneal route, the relatively slow decomposition of A gives it an advantage over B, for the local protein concentration would be rising only slowly. On the other hand, even a large intravenous dose of such a stable substance would be dispersed very quickly in the total blood-volume of the animal, while both B and C, more irritant owing to their acid decomposition products, would be partially localized in fibrin sheets along the blood vessels. Incidentally, the closeness of all the curves in Fig. 2 does suggest that the nature of the determinant group is fairly unimportant—a factor which, as mentioned earlier, was not under control.

The ineffectiveness of the small intraperitoneal dose of all three is understandable if there is simply not enough left of any of the substances to form effective antigen when the protein finally arrives; but the results of the small intravenous dose are rather more puzzling. For substance A, the small dose is just as effective as the large (compare sera 54 and 56 with sera 69 and 70), although the slope of the curves is curiously different. It is tempting to try to correlate this independence of dosage with the advantage held by substance A at high pH values—values which do not obtain in tissue fluids, but which might be approached in the cell. A small amount of substance which could survive and be taken up by a reticulo-endothelial cell might prove as effective an antigenic stimulus as much greater amounts of ready-conjugated homologous protein.

It may be observed in this connection that substance A is very much superior to the other two as a skin-sensitizing agent. The process of skin-sensitization entails a cellular factor, as the researches of Landsteiner and Chase have shown; whether the intracellular process is concerned with antigen or antibody, or both, is not yet decided. Recent work on antibody production has still not cleared up the question as to whether one cell takes up and "prepares" the antigen, against which another cell forms antibodies (Ehrich, 1946); but it seems to be agreed that a cell of the lymphocyte series is the most likely candidate for the latter function. Now the early cellular reaction in the dermis to an effective sensitizing substance, when given to the guinea-pig by intradermal injection, is definitely not mainly lymphocytic. Typically there is a marked proliferation of reticulum cells and a tissue eosinophilia (unpublished experiments). The staining-reaction of the cytoplasm of these cells is of the usual acidophilic kind, i.e. the cytoplasm is basic, in contrast to the cytoplasmic basophilia of the lymphocyte and plasma cell. Indeed, if the staining of the eosinophil granule is any indication of pH, this would provide an admirable locus for conjugation; but that is perhaps to speculate too far. Such a cell would anyway have in its cytoplasm a slightly basic environment for conjugation such as we have postulated.

Finally, some points may be worth considering in connection with the molar ratio of antigen to antibody in these sera. On the basis of a "valency" theory of the antigen-antibody reaction, it would seem reasonable to expect that the "equivalence-zone" ratio of antibody to antigen would be A_3G_2, i.e. the antibody being taken as divalent, and the antigen as functionally trivalent, which for the antigens here used is probably a minimum. Now assuming the molecular

weight of antigen (as gelatin) and antibody to be 18,000 and 120,000 respectively, the A_3G_2 ratio comes out as 10 : 1 on a weight basis; that is, one would expect 1 mg. of antigen to precipitate 10 mg. of antibody. The figure in column viii of Table II would then be 3. Taking a minimum value for the valency of antigen, and supposing the "equivalence point" to be the first antibody-excess tube, rather than the last antigen-excess tube, would both tend to make the figures in this column high; but in spite of this, the majority are between 1 and 2. This means that it takes a good deal more antigen to precipitate antibody than one would expect.

One can think of various possible explanations of this. It is possible, first, that the antibody contents of all the sera have been underestimated, although the underestimate would have to be rather gross to make so much difference. Secondly, the effect may be due to heterogeneity of the antigen (always possible when gelatin is the carrier)—a large proportion of the antigen-nitrogen added not being precipitable, either because its molecular weight is too low, or because the hapten groups are for some reason concentrated on a few of the molecules while others are unconjugated, or mono-conjugated, and acting as inhibitors. Thirdly, the effect may be a function of gelatin as such; if we abandon any considerations of valency, it is easy to imagine that a small, threadlike, soluble, amphoteric molecule like gelatin might be a less efficient specific precipitant in a colloid system than either a more normal protein, or even than a polysaccharide, with its battery of acidic groups.

SUMMARY.

1. Three substances known to combine chemically with the free amino-groups of proteins, namely, 2-phenyl-4-ethoxymethyleneoxazolone, benzyl chloroformate, and *p*-nitrobenzoyl chloride, have been tested for their capacity to provoke antibody formation on injection into rabbits.

2. Their antigenicity has been compared with their rate of hydrolysis and of reaction with amino-groups, as determined *in vitro*.

3. It is concluded that factors favouring antigenicity are a relatively slow rate of reaction and a high conjugation/hydrolysis ratio.

4. This suggests that conjugation with amino-groups occurring intracellularly, perhaps at an elevated pH, may play an important part in the formation of a potent antigen in the animal body.

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PHYSICAL PROPERTIES OF THE VIRUSES OF NEWCASTLE DISEASE, FOWL PLAGUE AND MUMPS.

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EXTENDED studies on the viruses of Newcastle disease, fowl plague and mumps were undertaken in order to obtain purified suspensions of these viruses and augment our knowledge of their physical properties. Advantage has been taken of the fact that these viruses, like influenza, may be assayed by means of the red cell agglutination test described by Hirst (1941). Furthermore, the electron microscope has been utilized to provide valuable, though not always encouraging, evidence on the degree of purification achieved, and to make possible a comparison of the sizes and morphologies of these viruses.

EXPERIMENTAL DETAILS.

1. *Strains of viruses used.*

Newcastle disease—"Herts" strain kindly supplied by Dr. J. M. Doyle. Ten-day hen's eggs were inoculated allantoically and the virus harvested after 36 hours' incubation at 37° C. The infective amniotic and allantoic fluids were studied separately.

Fowl plague—a Dutch strain also kindly supplied by Dr. Doyle. Ten-day hen's eggs were inoculated and incubated as for Newcastle disease virus (N.D.V.).

Mumps—Ender's strain EMA 41 kindly sent by Dr. H. Koprowski. This strain can be successfully passaged allantoically in hen's eggs. Eight-day eggs were inoculated and the infected allantoic fluid harvested after 4 days' incubation at 35° C.

2. *Assay of viruses.*

Two methods have been used: (1) The limiting infective dilution (L.I.D.) method in which serial ten-fold dilutions are suitably inoculated (0·05 c.c.) into hen's eggs of the appropriate age (Beveridge and Burnet, 1946). (2) The haemagglutination test in which the degrees of agglutination produced by serial two-fold dilutions of the virus are compared visually, using 0·25 per cent washed fowl red cells (Salk, 1944), or, photoelectrically, using 0·5 per cent cells (Hirst and Pickels, 1942).

3. *pH stability.*

The virus suspension was diluted one-hundredfold in Northrop–de Kruif buffers (Northrop and de Kruif, 1922) at 0° C. After being kept for chosen periods at this temperature each system was diluted ten-fold in saline broth and then titrated.

4. *Size estimation by filtration and centrifugation.*

The methods of ultrafiltration analysis employing gradocol membranes (Elford, 1931) and centrifugation analysis (Schlesinger, 1934; Elford, 1938) were applied in estimating the size and degree of homodispersity of the viruses

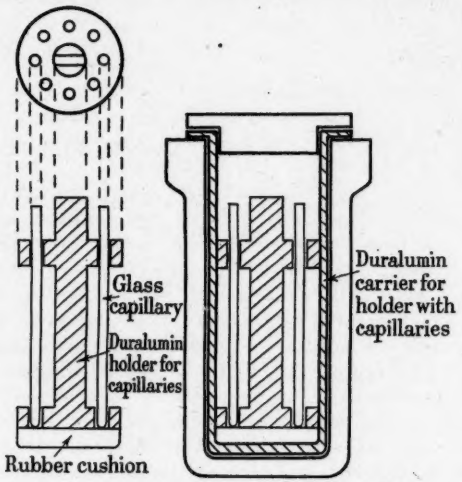


FIG. 1.—Holder for several capillary tubes.

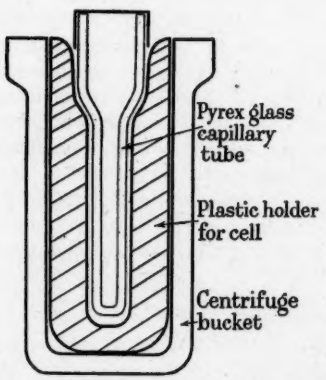


FIG. 2.—Another form of centrifuge tube.

in allantoic fluid. The use of capillary tubes for studying sedimentation with small quantities of material was found invaluable. Fig. 1 shows a holder for several tubes and the carrier which slides into a centrifuge bucket and is then capped. Fig. 2 illustrates yet another form of centrifuge tube we have used extensively in our experiments with purified virus concentrates.

5. *Density.*

The values of the densities of the viruses have been derived from their sedimentation rates in saline, sugar solutions (10 and 20 per cent) and bovine albumin 12·5 per cent of known densities and viscosities, by extrapolating for zero sedimentation (Sharp, Taylor, McLean, Beard and Beard, 1945).

6. *Methods of purification.*

Of the several methods investigated in the course of our studies two were the most successful: (1) Differential centrifugation using an air-driven quantity-type ultracentrifuge (Bauer and Pickels, 1936), and also a refrigerated centrifuge with high-speed head attachment (International Equipment Co.). Lusteroid tubes, sterilized by means of ultraviolet light, were used with these machines. Having concentrated the virus and reduced the volume to about 20 c.c. we conducted further fractionation in the "Ecco" centrifuge (20,000 g.), in which glass tubes may be used. (2) Adsorption and elution on and from the surface of washed fowl red cells. Precipitation of the virus on calcium phosphate followed by elution in a reduced volume (Salk, 1941) has proved useful in attaining a preliminary concentration and partial purification.

Centrifugation alternately at high and low speeds selected to retain the virus in a known fraction determined by its size and density is a method that has been widely used for plant and animal virus purification. Its application in this field has been well described recently in a review by Beard (1948). Adsorption of virus upon and its elution from the surface of washed fowl red cells, applicable to the group of viruses we are considering here, was first described by Hirst (1941, 1943), and has since been extensively used especially in work with influenza virus. Hirst showed that influenza virus was adsorbed and eluted from red cell stroma, but that the efficiency of adsorption was not as good as with intact cells. Our own experience, however, of using a suspension of the ghosts of laked red cells for adsorbing the virus has led us to the view that the value of the method is enhanced thereby. Virus may be adsorbed and eluted with excellent efficiency, the product from one such cycle of treatment containing so little protein as to be undetectable by the sulphosalicylic acid test. When the intact red cell is used, even with the greatest care, the eluate is invariably tinged with the colour of haemoglobin and protein is present in measureable amounts. The figures in Table I are typical for nitrogen and protein tests on routine eluates using (*a*) intact red cells and (*b*) suspensions of cell ghosts.

TABLE I.—*Adsorption and Elution Method Applied to Influenza "B" Virus in Allantoic Fluid.*

	Nitrogen mg./g.	Protein test with sulphosalicylic acid.
Allantoic fluid Lee "B" influenza	0·63	+++
Red cell supernatant after adsorption	0·44	+++
Red cell eluate	0·07	+
Laked cell supernatant after adsorption	0·41	+++
Laked cell eluate	Less than 0·01	—

Maybe the method we have used in preparing our laked cell suspensions (described below) is less damaging to the membranes than the two methods used by Hirst, namely the mechanical disruption of cells in a Waring blender and lysis by heating at 65° C. for half an hour. The procedure adopted in preparing the suspensions of cell ghosts has been to make a 1 per cent suspension of washed red cells and then to lake them with saponin following the method of Dounce and Lan (1943). Six efficient washings of the laked cells in 0·9 per cent saline-phosphate pH 6·7 suffice to remove the saponin, and the final volume of the suspension is adjusted to give 10 per cent ghosts. Actual counts were made in a haemocytometer cell. The extra time taken in preparing the laked as compared with the intact cell suspension is off-set by the fact that the former can be stored at 0° and used for several days at least. Some impairment of adsorbing capacity does occur with age, but several batches stored at 0° for two months showed only about 50 per cent reduction in efficacy.

7. *Preparation of the specimen for electron microscopy.*

The results presented here are for specimens mounted in the usual way on a collodion film support. A drop of 2 per cent "Necoloidine" in amyl acetate allowed to spread on water has yielded films of good strength and uniformity. The virus preparations have been suspensions in water or isotonic saline. The former were allowed to dry directly on the film, but with saline systems, after a period for adsorption of the virus on the film, the excess fluid was carefully removed. After air-drying, the film was washed several times with distilled water before finally being allowed to dry over phosphorus pentoxide. Both fixed (osmic acid) and untreated virus have been examined. Further, in order to enhance the contrast of the image produced the metal-shadowing technique of Williams and Wyckoff (1945) has been applied using gold and palladium. The latter metal has yielded more even depositions free from the inherent tendency to aggregate in the electron beam and give spurious effects, as sometimes shown with gold-shadowed preparations.

Fig. 4–8 were taken with a Siemens electron microscope at 85 K.V.

TABLE II.—*Physical Properties of Viruses of Newcastle Disease, Fowl Plague and Mumps*

Virus.	Strain.	pH stability.	Size by filtration (mμ).	Density 25° C.	Size by centrifugation (mμ).	Mean size by electron microscopy (mμ).
Newcastle disease	"Herts"	5·5–7·5	90–140	1·12 ± ·02	110–156	150 ± 50
Fowl plague	Dutch	7–9	75–115	1·14 ± ·02	90–110	83 ± 15
Mumps	Enders EMA 41	6·5–8·5	100–150	1·10 ± ·02	114–143	140 ± 30

EXPERIMENTAL RESULTS.

Data, established with the foregoing methods, on the physical properties of the viruses of Newcastle disease, fowl plague and mumps are summarized in Table II. They appertain to the viruses as developed in the amniotic and allantoic fluids of infected hen's eggs. Electron micrographs of the viruses purified from such fluids are given in Fig. 3–8.

Newcastle disease virus.

The infectivity of this virus was reduced by 90 per cent at pH 5·0 and likewise at pH 8·0 in 24 hours at 0° C. The range of stability found pH 5·5–7·5 is in accord with the findings of Moses, Brandly and Jones (1947). The filtration end-point for the virus was found to be 180 mμ., indicating a particle size 90–140 mμ. for the smallest form of the virus. Earlier experiments in this laboratory (Burnet and Ferry, 1934) with broth extracts of the lesions produced in the chorio-allantoic membrane gave filtration end-point 160 mμ. The filtration curves in our experiments suggested a considerable degree of polydispersity. This was borne out by centrifugation analysis on the virus in freshly harvested allantoic fluids and in purified preparations. A particle size ranging from 110–160 mμ. was indicated, based on the density value 1·12. Size estimations from electron micrographs of purified preparations of the virus gave a mean particle diameter 150 ± 50 mμ. (Table II) and an exceptionally wide spread (Fig. 11). The electron micrographs shown in Fig. 3–6 illustrate these facts, as well as the difficulty met in deciding upon the true morphology of Newcastle disease virus. Early in our work with purified concentrates of the virus we obtained pictures indicating gross pleomorphism—assorted round, sperm-shaped and bizarre forms in the same specimens (Fig. 3). Similar observations have been recorded by Cunha, Weil, Beard, Taylor, Sharp and Beard (1947), and Bang (1946, 1947). In a preliminary note Elford, Smiles, Chu and Dudgeon (1947) expressed doubt as to whether the sperm-like forms represented true phases in the morphology of the virus, and were inclined to the view that these and other bizarre structures were produced during the purification treatments and subsequent drying, through the virus being jacketed with a readily deformable, osmotically sensitive mucinous substance. Later studies have confirmed us in the latter view, and preparations in which exposure to excessive centrifugal fields was avoided and the suspensions were made in water or dilute electrolytes (M/200–M/450) showed generally round bodies (Fig. 4, 5, 6). These varied in size and apparent density, and there was evidence of the occurrence of two forms, one of which dried as a round opaque body and cast a well-defined shadow when metal-coated, and another which when dry was generally flat with a slightly raised centre. Such structures are to be seen in Fig. 5, 6. In some instances it appeared as though the more dense bodies were virus particles stripped of their mucinous jackets which could often be seen free as relatively flat structures (see Curves 1 and 2, Fig. 11, for analysis

EXPLANATION OF PLATES.

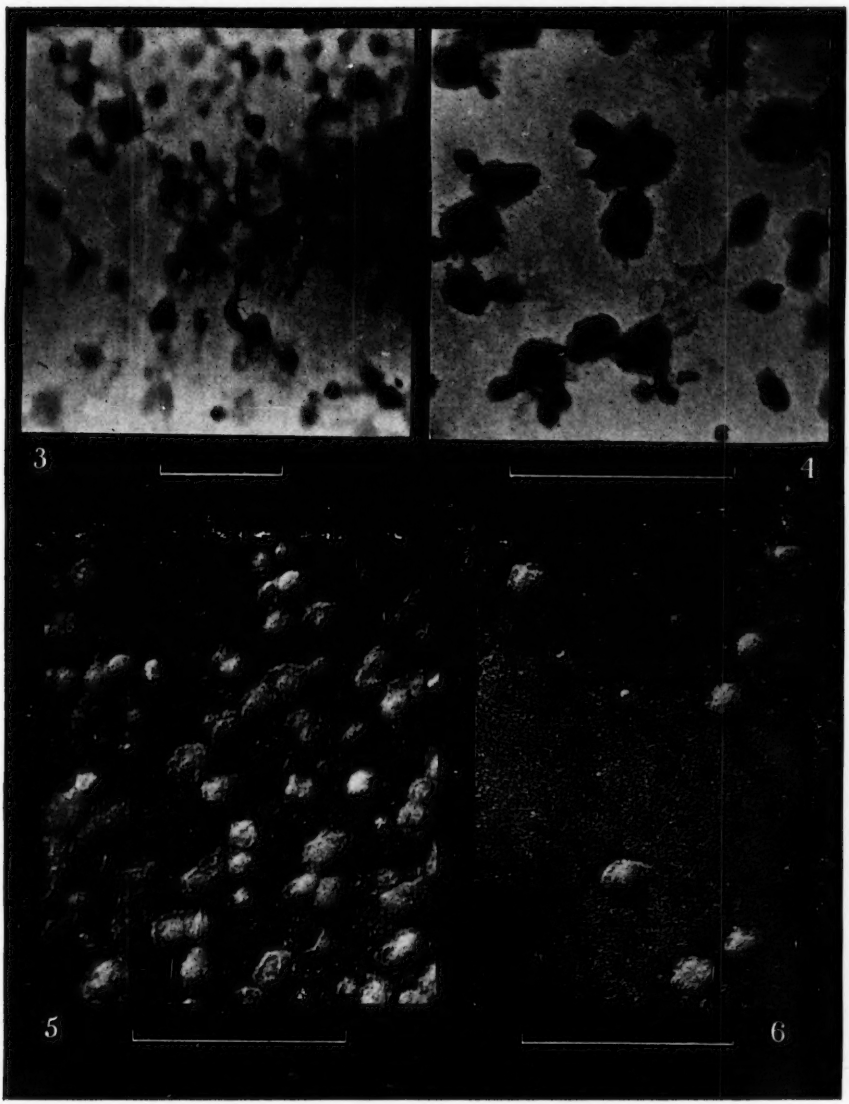
FIG. 3–6.—Newcastle disease virus from allantoic fluid. Fig. 3.—Centrifuged concentrate. (× 18,000.) R.C.A. microscope, 50 K.V. Fig. 4.—Eluted from calcium phosphate precipitate (Salk method), and then fractionated by centrifugation. Finally suspended in Ringer solution. (× 33,000.) Fig. 5.—Concentrated and purified by cycles of high and low-speed centrifugation. Palladium-shadowed. (× 32,000.) Fig. 6.—Purified by repeated cycles of alternately high and low-speed centrifugation. Palladium-shadowed. (× 32,000.)

The line beneath each picture = 1 micron.

FIG. 7.—Fowl plague virus from allantoic fluid. Partially purified by centrifugation and treatment with trypsin. Palladium-shadowed. (× 30,000.)

FIG. 8.—Mumps virus from allantoic fluid. Purified by cycles of high and low-speed centrifugation. Palladium-shadowed. (× 40,000.)

The line beneath each picture = 1 micron.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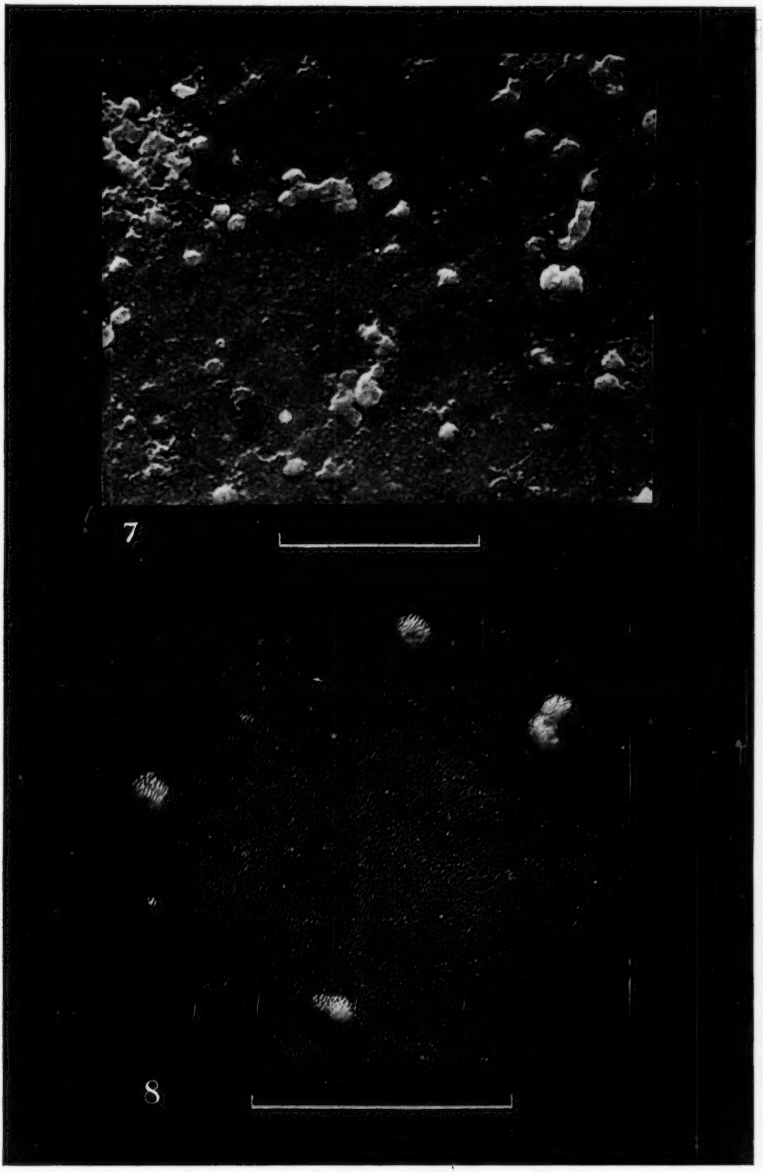
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of size distribution). This interpretation requires further support from alternative lines of evidence, particularly biochemical. Virus from the amniotic fluid was in general characterized by a greater proportion of the round dense bodies than in the allantoic fluid. This would provide an explanation of the more ready filtrability of virus in amniotic compared with the high losses incurred

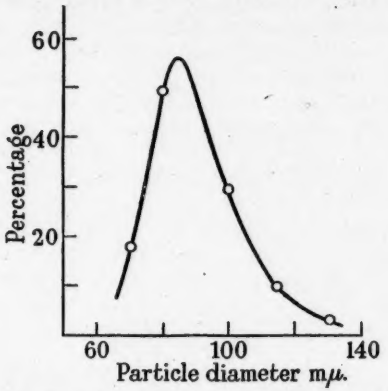

Fig. 9.—Spread of particle size of fowl plague virus.

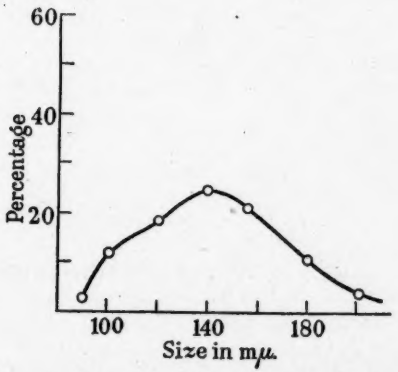

Fig. 10.—Spread of particle size of mumps virus.

when filtering virus in allantoic fluid. Our general conclusions regarding the morphological character exhibited by this virus appear to be in close accord with those of Bang (1948), although we could not subscribe to the view that it is pleomorphic in the degree shown by pleuropneumonia-like organisms.

We found the method of precipitation on and elution from calcium phosphates of value for concentrating and partially purifying this virus. Adsorption and elution from washed fowl red cells gave very erratic results and was not much used. Infected allantoic fluid with 0·33 saturated ammonium sulphate at 0°

yielded a precipitate, which, when redissolved in a small volume of distilled water and dialysed, was found to contain most of the virus present in the original fluid. Precipitations with 33 per cent alcohol at 0° destroyed the virus, and even 20 per cent alcohol resulted in a partial inactivation. Purified preparations of Newcastle disease virus were found to be very stable in water or dilute electrolyte media at pH 7. A preparation initially concentrated by Salk's phosphate precipitation method and then further fractionated with Ecco centrifuge yielded an intensely opalescent suspension in water. It was stored in a rubber-stopped Pyrex glass tube from September, 1946, to May, 1948, and tested periodically. There was not more than one tube difference ($\times$ 2) in the haemagglutination test, while the L.I.D. titre in eggs, initially just 10^{10}, was still 10^{9} after twenty

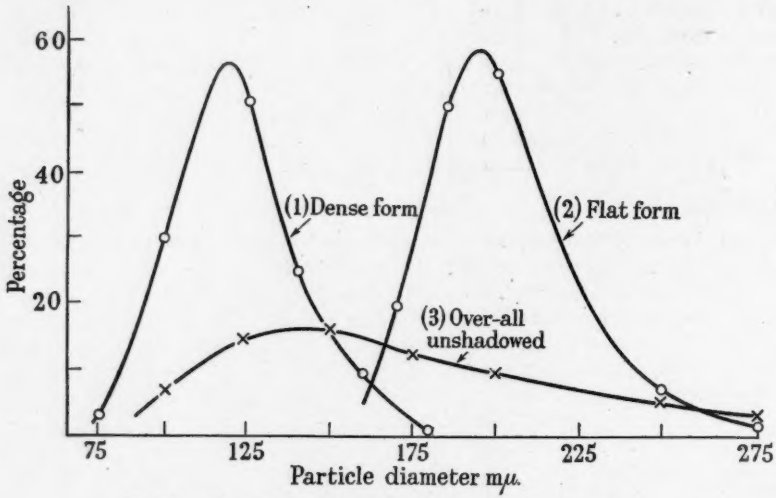

Fig. 11.—Spread of particle size of Newcastle disease virus.

months' storage. Examination of this and similar preparations with the electron microscope showed the virus bodies rather larger than when freshly prepared. and more transparent to electrons with attendant loss of contrast in the image (Fig. 11, Curve 3).

In studies on the action of enzymes on the purified Newcastle disease virus it was found that at pH 8·2 trypsin (crystallized) did not inactivate the virus in 20 hours at 37°, and the haemagglutinating activity was actually slightly increased. With papain (partially purified), on the other hand, inactivation definitely did occur at pH 7, although the haemagglutination titre was little affected.

Fowl plague virus.

This virus lost 90 per cent of its infectivity at pH 6 and at pH 9·9, the range of maximum stability being pH 7–9. This is somewhat higher than in the case of Newcastle disease virus—a result in agreement with the work of Pyl (1938) and Moses, Brandly and Jones (1947). The filtration behaviour of this Dutch

strain of fowl plague virus in allantoic fluid was different from that characterizing the "Brescia" strain in pericardial fluid studied by Elford and Todd (1933). Whereas the latter strain yielded filtration curves suggesting a high degree of homodispersity for the virus and a sharp end-point at 120 mμ, the present strain in allantoic fluid filtered with considerable loss over a wide range of porosity 0·2–0·7 μ, with an end-point at about 150 mμ. The size of the smallest form would therefore be deduced as 75–115 mμ., compared with 60–90 mμ. for the Brescia strain, and there was a strong indication that the virus was far from being homodisperse. Centrifugation analysis on 0·7μ filtrates of allantoic fluid and purified virus gave a particle diameter 90–105 mμ. for the density value 1·14. This did not point to any appreciable polydispersity. Similarly electron micrographs of purified virus prepared by adsorption and elution from washed fowl red cells, combined with fractional centrifugation in the Ecco centrifuge, indicated a mean particle size 83 ± 15 mμ. for the dried specimen and an overall spread in size 60–140 mμ. (Fig. 9). In shape the elementary virus bodies appeared round (Fig. 7) and they exhibited poor contrast, and could only be satisfactorily studied with the aid of the shadow-casting technique.

Trypsin, acting on partially purified fowl plague virus at pH 8 for 30 minutes at 37° C. did not affect the haemagglutination titre.

Mumps virus.

The pH range of stability for this virus at 0° C. was found to be pH 6·5–8·5. The infectivity in eggs was reduced by 90 per cent in 24 hours at pH 6 and at 9·0. The haemagglutinin showed a maximum stability at pH 7 ± 0·5. Recently Weil, Beard and Beard (1948) have reported the optimum pH stability range for this same strain of mumps virus to be 5·65 to 7·9. The filtration end-point for the virus in allantoic fluid diluted with Hartley broth was 200 mμ., indicating the size to be 100–150 mμ. Habel (1945) found that mumps virus from chick embryo was retained by 380 and 250 mμ. membranes. In order to obtain 250 mμ. filtrates consistently positive it is, in our experience, essential to have the virus in a medium containing broth with L.I.D. at least 10^6. We have made five centrifugation analyses of infected allantoic fluid, using the R.C.A. test for virus assay, and the particle diameters, calculated for the virus density value 1·10, were 143, 114, 138, 140 and 116 mμ. The electron microscope pictures of purified mumps virus showed an appreciable spread in particle size from 85 to 200 mμ. (Fig. 10), the mean size being 140 ± 30 mμ. Fig. 8 shows a micrograph of a palladium-shadowed specimen. In general the virus dried as a dome-shaped body, as indicated by the shadow, but the largest forms were relatively flat. Wiel, Beard, Sharp and Beard (1948) describe formolized virus being spherical and flattened in the electron microscope and having particle diameter 190 ± 42 mμ.

Mumps virus is poorly adsorbed from allantoic fluid by washed fowl red cells, or laked cells. The adsorption and elution method of purification was not readily applicable, and our purified preparations of the virus have been made by the fractional centrifugation procedure. When concentrated by centrifugation and resuspended in 0·9 per cent saline phosphate at pH 6·7 the adsorption was much improved. Mumps-infected allantoic fluid was successfully dialysed at 0° against M/250 citrate-phosphate buffer of pH 7·2 without decrease of R.C.A. titre. Trypsin at pH 8·5 was found to have partially inactivated the virus

after 18 hours at 37°, the effect being in addition to the adverse influence of this degree of alkalinity on the virus at 37°.

DISCUSSION.

The figures for the particle sizes of the three viruses here under investigation have been derived from three independent methods, filtration analysis, centrifugation analysis and electron microscopy. Each method has its inherent difficulties and shortcomings, but there is general agreement among the size values indicated. It is well to remember that the methods of filtration and centrifugation analysis are applied to the study of virus in fluid suspensions as freshly harvested, and respectively give evidence on the size of the smallest phase and a figure for the average based on an assumed uniformity of spherical shape. In the electron microscope examination is made of a dried purified virus preparation, which may or may not be representative according as to whether, during the purification treatment, serious loss of large or small forms has been avoided or not. Allowing for the uncertainty as to the effects of desiccation on biological material, electron microscopy can give direct evidence regarding the morphology and general size distribution in the specimens examined. Furthermore, the electron microscope may be used to provide invaluable information on the degree of purification achieved following successive manipulations, as indicated by the predominant occurrence of particles of particular size and shape as the specific activity increases. Applied, during our present studies, to preparations obtained by fractional centrifugation, the method has brought home to us most forcibly the difficulty of obtaining any one of the viruses in suspensions that can with fair approximation be termed pure. The problem varies from virus to virus, and according to the particular source of virus, e.g. whether tissue suspension, amniotic or allantoic fluid, vesicular fluid, etc. The spectrum of particle size distribution curves will vary enormously. In some favourable cases the peak for virus may be reasonably well isolated, and in others there may be considerable overlapping of the curves. This will depend not only on the particles occurring in normal tissues and fluids, but will certainly vary with the type and severity of the interaction of the infective agent with the host cell. Accordingly each virus demands special study, and in the present investigations we have exploited most of the well-known methods for concentrating and purifying viruses. The scale on which we have been able to work has permitted only a small volume of purified virus to be obtained in each experiment. Consequently, any extensive biochemical tests have been precluded.

Mention has been made of our use of carefully prepared suspensions of the ghosts of laked fowl red cells for the adsorption and elution of viruses. This has formed the basis of a new technique whereby the viruses of this group, adsorbed on the cell membrane, may be conveniently studied in the electron microscope (Dawson and Elford, 1949).

SUMMARY.

New data on the physical properties—pH stability, density, and particle size by filtration analysis, centrifugation analysis and electron microscopy—of the viruses of Newcastle disease, fowl plague and mumps are presented.

The value attaching to the use of carefully prepared suspensions of the ghosts of laked fowl red cells for obtaining purified concentrates of certain viruses by the adsorption and elution method is emphasized.

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IMMUNIZATION AGAINST LOUPING-ILL. IMMUNIZATION OF CATTLE.

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ALTHOUGH louping-ill is primarily a disease of sheep, cattle grazing on infected hill land not uncommonly become infected. The susceptibility of cattle is likely to become of increasing economic importance since the grazing of hill land with cattle is being encouraged as part of the national agricultural policy. In view of the successful control of louping-ill in sheep by a single-dose method of vaccination with a formalinized vaccine, veterinary surgeons practising in endemic areas have attempted to protect cattle with the same vaccine. Some have had encouraging results, but on the other hand there have been many complaints that this vaccination has failed to protect. No field trial of the vaccination of cattle has been reported and it is for these reasons that an experimental investigation was undertaken.

It appeared likely that certain general principles, which experience has shown determine the possibility of protecting animals with inactivated vaccines against virus infections, would apply to the immunization of cattle against louping-ill; the validity of these principles has already been demonstrated for the immunization of sheep and mice against louping-ill (Edward, 1947). Formalinized vaccines can be prepared either from the tissues of the same species of animal as that which is to be immunized, or from those of a heterologous species. Homologous tissue vaccines can produce a satisfactory level of immunity even when the concentration of virus before inactivation is relatively low. Sheep tissue vaccines can protect sheep against louping-ill even when the original tissue suspension was infective only to a limiting dilution of 10^{-4}. On the other hand, general experience in vaccination against virus infections has suggested that much higher concentrations of virus antigen are essential in heterologous tissue vaccines, original virus titres exceeding 10^{-7} usually being necessary.

Although in attempting the protection of cattle against louping-ill it was necessary to consider both types of vaccine, the results of an earlier preliminary experiment suggested that an homologous tissue vaccine would not be satisfactory. An 11 days-old calf was inoculated intracerebrally with a strain of louping-ill virus which had been maintained by passage in sheep. It developed typical signs of louping-ill after 5 days and was killed on the 6th day when it was completely paralyzed. A 10 per cent suspension of its brain in saline was infective for mice to a limiting dilution of only 10^{-2}. Unless higher concentration of virus than this could be obtained it appeared unlikely that the brains of cattle would provide satisfactory vaccines. It is possible that higher titres might be obtained if the virus were passaged a number of times in cattle or if another strain, particularly one recently isolated from a field infection, were used.

However, it did not appear justifiable to explore this possibility as it would have necessitated the sacrifice of a number of cattle, perhaps in vain.

Immunization of cattle with formalinized heterologous tissue vaccines was therefore attempted. Tests were made with the ordinary sheep tissue vaccines issued for the protection of sheep. The concentration of virus antigen in these vaccines is such that before inactivation they are infective for mice at a limiting dilution of between 10^{-4} and 10^{-5}. For the reasons already stated it was thought likely that these titres might be too low for the effective protection of a heterologous species. Trial was therefore also made of vaccines prepared from the brains of infected mice in which at least a hundred-fold greater concentration of virus can be obtained.

TECHNICAL METHODS.

The methods used for preparing vaccines from the brains of infected mice and from the brains, cords and spleens of infected sheep have been described in a previous paper, which also gives details of the strains of virus (Edward, 1947). For challenging vaccinated cattle and for infecting sheep for vaccine production a strain of virus which had been maintained by passage in sheep was employed. A mouse-adapted strain was used for preparing a vaccine in mice. Vaccines were made from the tissues of infected sheep and mice, harvested when the animals were paralyzed; 10 per cent suspensions in buffered saline (M/50 phosphate in 0·68 per cent saline at pH 7·6) were centrifuged at 2000 r.p.m. for 10 minutes and the supernatants were inactivated with 0·2 per cent formalin.

Serological response in vaccinated heifers.

The effects of vaccination with mouse brain and sheep tissue vaccines were tested in a group of 7 heifers between 9 and 15 months of age. Three animals received two subcutaneous inoculations, each of 5 ml., at an interval of 14 days of a mouse brain vaccine, which had had before inactivation an $L.D._{50}$ titre of $10^{-8\cdot2}$. Another two animals had two doses of 10 ml. at a similar interval of a sheep tissue vaccine prepared from a 10 per cent suspension infective to a limiting dilution of $10^{-4\cdot0}$. A third group of 2 animals received a similar course of inoculations with 10 ml. of a sheep tissue vaccine, prepared as a 2·5 per cent suspension from the same batch of infected tissues. Samples of blood, withdrawn from each animal before the first inoculation and again 10 days after the second dose, were examined for the development of neutralizing and complement-fixing antibodies.

The results are shown in Table I. To economize in mice the preinoculation samples of serum from each group were pooled for the titration of neutralizing antibody. None of the pooled samples showed any neutralization. After vaccination the sera of two of the three heifers given mouse brain vaccine contained neutralizing antibodies; in one the neutralization index was 250 and in the other 50. Only one of the four heifers, which received sheep brain vaccine, developed neutralizing antibodies (neutralization index of 25). Although the sera of several of the heifers showed a significant degree of fixation of complement after vaccination, the results were of doubtful significance as a few of the preinoculation

TABLE I.—*Serological Response in Vaccinated Heifers.*

Animal.	Method of immunization.	Neutralization Index. Before immunization.	Neutralization Index. After immunization.	Complement Fixation Test. Before immunization. Dilutions of serum. ½	¼	Complement Fixation Test. After immunization. Dilutions of serum. ½	¼	1/8
1	Two 5 ml. doses of 10% mouse brain vaccine at interval of 14 days	0	250	+++	++	++++	++++	+++
2	,,		50	—	—	—	—	—
3	,,		0	+++	+	+++	+	—
4	Two 10 ml. doses of 10% sheep tissue vaccine at interval of 14 days	0	0	—	—	+	±	—
5	,,		0	—	—	+	±	—
6	Two 10 ml. doses of 2·5% sheep tissue vaccine at interval of 14 days	0	25	+	—	+++	+	±
7	,,		0	—	—	±	—	—

samples also fixed complement. This could be explained by the tests not being sufficiently specific or by clinical or subclinical infection of the heifers previously with louping-ill. The animals had been bred on the Field Station, where louping-ill has been continually under investigation in sheep and it is possible that they may have come into contact with the virus. The preinoculation serum of heifer 1, which gave a significant degree of fixation of complement before vaccination and had the highest neutralization index afterwards was shown, when tested individually, not to contain neutralizing antibodies. However, although the results of the neutralization tests were clear-cut and showed that neutralizing antibodies appeared in the sera of certain animals after vaccination, whereas they were absent previously, the doubts raised by the results of the complement-fixation tests suggested that too much reliance could not be placed on the experiment which ought to be repeated.

Challenge of vaccinated cattle with live virus.

To continue the investigation, 14 young animals, 11 heifers and 3 steers, were purchased. Five animals were vaccinated with mouse brain vaccine, three receiving two 5 ml. doses at an interval of 14 days, and two only one dose. Another group of three animals had two 10 ml. doses at an interval of 14 days of the same 2·5 per cent sheep tissue vaccine which was used in the first experiment. Two further animals were similarly vaccinated with the sheep tissue vaccine except that 28 days elapsed between the injections.

Neutralizing antibodies were absent from samples of serum obtained before vaccination and 10 days after the second dose from the three animals which had two doses of mouse brain vaccine and from the three vaccinated with two doses of sheep brain vaccine at an interval of 14 days. The results of complement fixation tests on the same sera were indefinite ; the three animals vaccinated with mouse brain vaccine gave doubtful positive fixation after vaccination, whereas the preinoculation samples were negative. Two of those vaccinated with sheep brain vaccine had no complement-fixing antibodies either before or after vaccination ; serum from the third (No. 12) gave incomplete fixation both before and after.

All the vaccinated together with control non-vaccinated animals were challenged by a method similar to that found satisfactory for testing the immunity of sheep (Edward, 1947). Each animal was inoculated subcutaneously with 5 ml. of a 10 per cent suspension of infected sheep brain, the inoculum being shown by mouse titration to contain between 10^5 and 10^7 M.L.D. of virus. Three days later 4 ml. of a 2 per cent suspension of commercial starch in saline was injected intracerebrally under chloroform anaesthesia. In the first two animals the injection was made posterior to the base of the horns ; subsequently a position anterior to the horns and to one side of the mid-line was preferred. After trephining the skull the inoculum was introduced through a needle 3 in. long, which was felt to enter the brain after traversing the frontal sinus. The animals were challenged at an interval after vaccination which varied between 20 and 46 days (Table II). They were kept under close observation for at least 14 days and their temperatures were recorded twice daily.

The first non-vaccinated animal to be challenged was pyrexial between the 10th and 15th days with a maximum of 104·6° on the 13th day. It became

TABLE II.—*Results of Challenging Vaccinated Cattle.*

Animal.	Method of vaccination.	Interval in days between last inoculation and challenge.	M.L.D. in challenge dose.	Presence of virus in blood on 3rd day.	Pyrexia after 5th day.	Signs.
18	Not immunized	—	10^7	?*	Pyrexia 10th to 15th days with peak of 104·6° on 13th day	Ill; muscular twitching; eyes "staring." Sluggish and frequent trembling. Ataxia.
20	ditto	—	10^5	+	102·8° once on 8th day	Ill; muscular twitching; Ataxia.
9	Two doses (5 ml.) mouse brain vaccine at interval of 14 days	20	10^7	—	Apyrexial	Unaffected.
8	ditto	31	10^7†	—	103·2° once on 7th day	No abnormal signs.
10	,,	34	10^6	—	Apyrexial	Unaffected.
14	1 dose (5 ml.) mouse brain vaccine	28	10^6	?*	Apyrexial	Unaffected.
15	ditto	28	10^6	+	Up to 105° on 7th day	No abnormal signs.
13	Two doses (10 ml.) sheep tissue vaccine at interval of 14 days	33	10^6	—	Pyrexia 7th to 9th days. Maximum of 104·8°	No abnormal signs (had a broken horn).
11	ditto	46	10^5	—	Apyrexial	Unaffected.
12	,,	46	10^5	—	103·2° once on 8th day	No definite abnormal signs.
16	Two doses (10 ml.) sheep tissue vaccine at interval of 28 days	34	10^6	+	Apyrexial	No abnormal signs.
17	ditto	34	10^6	—	Apyrexial	Unaffected.

* Indefinite result owing to non-specific deaths among mice. † Mouse passage strain of virus used for challenge.

obviously ill. It was sluggish, trembled and had muscular twitching; its eyes were "staring." Later, definite ataxia was noted. It made a complete and comparatively rapid recovery. A second control animal remained apyrexial except for reaching a temperature of 102·8° once on the 8th day, but it also became ill with muscular twitching and ataxia. Recovery was complete after a few days. By inoculating mice, virus was demonstrated in a sample of defibrinated blood taken at the time starch was injected. In order to determine whether the third day was the most suitable for injecting the starch, in another control heifer starch was injected 7 days after the inoculation of virus; it remained unaffected. A further control animal, inoculated with the mouse passage strain of virus but not injected intracerebrally with starch, was also unaffected.

Using the method of challenge, in which the inoculation of virus was followed three days later by the intracerebral injection of starch, none of the vaccinated animals became ill or showed nervous symptoms, such as were shown by both controls. This suggests that some protection had been provided by vaccination, although pyrexia or the demonstration of virus in the blood in the third day in certain of the animals showed that protection was not always complete (Table II). Of the three animals vaccinated with two doses of mouse brain vaccine, two were apparently unaffected and remained apyrexial except for the two days following the intracerebral injection, when all the animals had a fever, presumably due to the operative interference. The third animal (No. 8) showed no abnormal signs but its temperature rose to 103·2° once on the 7th day. One of the two animals which had only one dose of the mouse brain vaccine was unaffected, but the other (No. 15) had a temperature which reached 105° on the 7th day; the demonstration of virus in the blood on the third day also showed that in this animal multiplication of virus had not been prevented. Five animals were vaccinated with two doses of sheep brain vaccine. Two were apparently unaffected. One of the other animals (No. 13) was pyrexial for 3 days, commencing on the 7th day and reaching a maximum of 104·8°. Unfortunately the horn of this animal was broken when it was being caught for the intracerebral inoculation. Although it was considered at the time that this injury, which later became septic, did not account for the pyrexia, the possibility could not be excluded. The temperature of another animal (No. 12) was raised to 103·2° on one occasion only. The fifth animal (No. 16) was apparently unaffected, but virus was demonstrated in its blood on the 3rd day.

DISCUSSION.

It has been shown that in normal cattle an attack of louping-ill can be produced by the subcutaneous inoculation of virus, provided that the brain is traumatized by starch injected intracerebrally 3 days later, at a time when virus is circulating in the blood. Earlier experiments in sheep had demonstrated that encephalitis could be produced with regularity in this species when passage of virus through the blood-brain barrier was facilitated by the injection of starch (Edward, 1947). However, whereas in non-immune sheep the encephalitis was always progressive and fatal, the disease in the two non-vaccinated heifers was mild and recovery was rapid, although definite ataxia was noticeable for a few days. The mildness of the disease in cattle may have been due to the strain of

virus, which had been maintained for a long time in sheep. It is possible that a strain of virus isolated from a field infection in the bovine, especially if it were passaged in cattle, might prove more virulent. The lack of virulence of the strain used is unfortunate, because the experiments are open to the objection that different results might have been obtained with a more virulent strain. Vaccination which could protect against the weakly virulent laboratory strain might prove ineffective under field conditions against a more virulent one. It is also possible that use of a more virulent strain might have revealed greater differences between the methods of vaccination tested, particularly between the mouse brain and sheep tissue vaccines.

Under the conditions of test both types of vaccine produced some protection, for none of the vaccinated animals showed ataxia or other nervous manifestation, such as were exhibited by both control animals. The best results were obtained with two 5 ml. inoculations of mouse brain vaccine. One dose was not sufficient. It has already been pointed out that from theoretical considerations a superiority of this vaccine over sheep tissue vaccine had been expected. For use in the field a formalinized mouse brain vaccine probably provides the more efficient antigen and is likely to provide adequate protection. However, it is obviously much more difficult and expensive to prepare and, if a sufficient degree of protection were provided by a sheep tissue vaccine, it would be preferable to use the latter. For the reasons already given the laboratory tests, which showed that it gave partial, although incomplete, protection, do not provide a definite answer as to whether it would prove reliable in the field. It is possible that by using two 10 ml. doses with at least 14 days' interval between them some degree of protection might be provided.

It is to be noted that a degree of protection was provided by vaccination in the absence of demonstrable serum antibodies. Antibodies are also absent from the sera of sheep immunized by the customary one dose method of vaccination, although they usually appear after two doses (Wilson, 1947, personal communication). Their absence in cattle after two doses emphasizes that reliance cannot be placed on only one dose. The results of the serological tests confirm those of the challenge experiments and suggest the superiority of the mouse brain vaccine.

SUMMARY.

Attempts were made to protect cattle against louping-ill by vaccination with formalinized vaccines prepared from infected mouse brains and from infected sheep tissues.

Measurement of antibody response did not provide adequate information regarding the effectiveness of the vaccines.

Vaccination provided a degree of protection, which was not always complete, against experimental challenge by a method in which live virus was inoculated subcutaneously followed three days later by an intracerebral injection of starch.

The mouse brain vaccine proved the better antigen; it was necessary to give two 5 ml. doses at an interval of 14 days.

Some degree of protection was provided by two 10 ml. doses of sheep tissue vaccine, but, owing to the low virulence for cattle of the strain of virus used in

the experiments, it was not possible to decide whether this vaccine would provide a sufficient degree of immunity under field conditions.

I wish to thank Mr. S. L. Hignett, B.Sc., M.R.C.V.S., for his advice and assistance in the operations on the cattle. I am also indebted to Mr. W. A. Fitzgerald for his technical assistance.

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SURVEY OF PAPERS.

HARTROFT finds that the first renal lesion in both weanling and young adult rats fed on a choline-deficient diet is the intracellular formation of small droplets of fat in the proximal convoluted tubules of the cortex. In weanling rats the droplets appear rapidly, producing tubular ischaemia and necrosis, whereas in young adult rats the droplets are formed later, producing atrophic tubular lesions of the "disuse" type (p. 483).

BARNES describes the development of a black pigmentation of the duodenal mucosa in the rat after subcutaneous injection of tannic acid. The staining is due to a deposit of iron-containing pigment on the adventitial membrane of the capillaries in the duodenal villi (p. 495).

MCCARTHY AND DOWNIE show that there is a close immunological relationship between virus strains of variola major, alastrim, vaccinia, cowpox and ectromelia by neutralization tests on the chorio-allantois of chick embryos with antisera prepared in fowls by the repeated injection of suspensions of these virus strains (p. 501).

GODLOWSKI finds that the anaphylactogenic protein is present in eosinophils from the anaphylactic peritoneal exudate of guinea-pigs sensitized to protein antigens. On the basis of these findings he suggests a theory of the role of eosinophils in allergic processes (p. 511).

ADA AND FULTON find that, during the course of visceral leishmaniasis in the golden hamster, there is a decrease in serum albumin and an increase in α-globulin, accompanied by an increase in phospholipids, cholesterol and neutral fat. These changes appear to be due to the activity of Leishman-Donovan bodies, and not to the renal lesions invariably associated with this infection (p. 524).

ISAACS finds that neutral mixtures of influenza A virus with their appropriate antisera can be reactivated *in vitro* by the addition of homologous, heat-inactivated virus. In the chick embryo reactivation can be produced by injecting homologous inactive virus 5–45 minutes before injecting a neutral serum-virus mixture. A possible explanation of the results is given based on the assumption that reactivation occurs according to the law of mass action (p. 529).

BAILIE AND IRVING have produced a narrow strip of calcification in the metaphyses of rachitic rats by starvation, or adding phosphate to the rachitogenic diet for a short period, and have then investigated the changes in their metaphyses. They find that the epiphyseal cartilage continues to proliferate but is kept at constant width by "renewed cartilage absorption," and growth occurs at the epiphyseal cartilage-metaphyseal junction, where fresh osteoid is laid down as the process of "renewed cartilage removal" takes place (p. 539).

DICK, SMITHBURN AND HADDOW have isolated a hitherto undescribed virus from two rhesus monkeys, from mosquitoes and from a mongoose in Mengo District in Buganda, Uganda. It has been named Mengo encephalomyelitis virus, and is immunologically distinct from 19 other neurotropic viruses (p. 547).

DICK finds that Mengo encaphalomyelitis virus is pathogenic to monkeys, mice and guinea-pigs but not to rabbits, and describes the pathological lesions induced by the virus. The virus is about 10 mμ in size, and is remarkably resistant to heat and certain chemical substances (p. 559).

GELL, HARINGTON AND MICHEL have investigated three simple chemical compounds and have compared their antigenicity in rabbits with their rate of hydrolysis and of reaction with amino-groups, as determined *in vitro*. They conclude that factors favouring antigenicity are a relatively slow rate of reaction and a high conjugation/hydrolysis ratio (p. 578).

ELFORD, CHU, DAWSON, DUDGEON, FULTON AND SMILES have studied the physical properties of the viruses of Newcastle disease, fowl plague and mumps by filtration analysis, centrifugation analysis and electron microscopy. They emphasize the value of the use of carefully prepared suspensions of the ghosts of laked fowl red cells for obtaining purified concentrates by the adsorption and elution method (p. 590).

EDWARD, continuing his studies in immunization against louping-ill, finds that some degree of protection is provided in cattle by subcutaneous inoculation with formalinized vaccines prepared from infected mouse brains and from infected sheep tissues (p. 600).

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